Universidade de Lisboa
Faculdade de Ciências

Departamento de Biologia Animal

Unravelling the regulation of Dll4 expression and its function during embryonic neurogenesis

Pedro Miguel Branco Barbacena

Dissertação
Mestrado em Biologia Evolutiva e do Desenvolvimento

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Unravelling the regulation of Dll4 expression and its function during embryonic neurogenesis

Dissertação orientada por:
Catarina Ramos, PhD, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa
Maria Gabriela Rodrigues, PhD, Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa

Pedro Miguel Branco Barbacena

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ABSTRACT

During spinal cord development, Notch signalling regulates both the maintenance of progenitors and neuronal specification. In most spinal cord domains, a single Notch ligand is expressed however in the V2 domain two ligands (*Dll1* and *Dll4*) are expressed. It is not known how *Dll4* expression is regulated and how *Dll4*-mediated Notch signalling has a role in the specification of V2 interneurons (V2a, V2b and V2c) that are generated from V2 progenitors.

To investigate how *Dll4* expression is regulated, we have focused on three proneural bHLH genes (*Mash1*, *Ngn1* and *Ngn2*), expressed in V2 progenitor cells. We characterized *Dll4*-expressing cells, by double RNA *in-situ* hybridization with each of the proneural genes. Our results revealed that *Dll4*$^{+}$ cells can coexpress all three proneural genes but to different extents. Next, we have overexpressed each proneural gene (singly and in combination) in the chick neural tube, using *in ovo* electroporation. Embryos were tested in terms of: *Dll4* expression and V2 cell-type specification.

Although electroporation of either *Mash1* or *Ngn1* leads to downregulation of *Dll4* expression in the V2 domain, only NGN1 is capable of inducing ectopic *Dll4* expression. Moreover, both double electroporation of *Mash1* and *Ngn1*, or *Mash1* and *Ngn2*, represses *Dll4* expression in the V2 domain, while leading to ectopic expression in other ventral spinal cord domains.

Concerning cell-type specification, electroporation of *Mash1* represses both V2a and V2b cell fate. Electroporation of either *Ngn1* or *Ngn2* increases the number of V2a INs, while repressing V2b INs. Double electroporation of *Mash1*/*Ngn1*, *Mash1*/*Ngn2* or *Ngn1*/*Ngn2* increases the number of V2a INs and represses V2b fate.

Together, the results presented in this thesis show that MASH1 and NGN1 can repress *Dll4* expression and that this repression correlates with alterations in V2 IN specification. Our results indicate that NGN2 can affect V2 specification, although it does not regulate *Dll4* expression.

Keywords: Neurogenesis, Spinal Cord, V2 domain, Notch Signalling, Delta-like 4, Proneural Proteins.

RESUMO

Um dos mais complexos sistemas biológicos é o Sistema Nervoso Central que é constituído por um elevado número e variedade de células, como neurónios e células da glia. Estas células são produzidas durante o desenvolvimento embrionário no momento e posição correctos de modo a interagirem entre si e formarem circuitos funcionais. Durante o desenvolvimento embrionário da espinal medula, vários interneurónios excitatórios e inibitórios são gerados, sendo o balanço entre eles estritamente regulado. No entanto os mecanismos moleculares subjacentes a esta diversidade celular ainda não são totalmente conhecidos.

A via de sinalização Notch desempenha um papel essencial durante o desenvolvimento do Sistema Nervoso Central. Esta via de sinalização é um sistema de comunicação célula-a-célula, de contacto directo entre duas células vizinhas, através de duas proteínas membranares: o receptor Notch e os seus ligandos (*Delta*, *Serrate* ou *Lag*-2, dependendo do organismo). Sempre que um ligando de uma célula se liga ao receptor de outra célula, uma série de clivagens proteolíticas são desencadeadas, o que, em última instância, leva à libertação do domínio intracelular do receptor Notch (NICD). O NICD é translocado para o núcleo onde se associa ao CSL e ao *Mastermind*, activando a transcrição de genes alvo, como os genes *HES*. Na ausência de NICD, a proteína CSL actua como repressor transcricional dos mesmos genes.

Uma das funções da via de sinalização Notch é a capacidade de manutenção de progenitores neurais, durante a neurogénese, impedindo uma diferenciação prematura massiva de todos os progenitores. A decisão de uma célula permanecer como progenitor, ou de se diferenciar, é controlada pelo balanço de dois tipos de factores de transcrição: as proteínas proneurais, que promovem a diferenciação; e as proteínas HES, que reprimem a diferenciação neural. Uma célula que, estocasticamente expresse elevados níveis de proteínas proneurais vai diferenciar-se. Normalmente as proteínas proneurais activam a expressão dos ligandos do Notch, o que leva a que esta célula expresse elevados níveis de ligandos, activando a via de sinalização Notch nas células vizinhas. Esta activação leva à expressão de elevados níveis de proteínas HES, sendo que estas células são mantidas como progenitores.

Assim, a célula que expressa o ligando diferencia-se em neurónio mas simultaneamente assegura que as células vizinhas se mantenham como progenitores. Visto que a neurogénese ocorre durante uma larga janela temporal, a manutenção de uma população de progenitores permite que estas células sejam expostas a diferentes estímulos e, como tal, se diferenciem em diferentes neurónios durante o

desenvolvimento, assegurando uma grande variedade celular ao nível do Sistema Nervoso Central.

A espinal medula, ao longo do seu eixo dorso-ventral, pode ser dividida em onze domínios molecularmente distintos (seis deles dorsais e cinco ventrais). Estudos recentes mostram que a via de sinalização Notch está envolvida na especificação de células neurais, nomeadamente de interneurónios no domínio V2 da espinal medula. Neste domínio, três tipos de interneurónios diferentes: V2a, V2b e V2c, são gerados a partir de progenitores comuns. Na ausência da sinalização Notch, apenas os interneurónios V2a são gerados, o que significa que a sinalização Notch é necessária para a produção dos interneurónios V2b. Os interneurónios V2c foram recentemente identificados. Curiosamente, nesta fase do desenvolvimento, este é o único domínio onde se sabe que diferentes interneurónios são simultaneamente produzidos e o único domínio onde dois ligandos da via Notch são expressos: *Dll1* e *Dll4*. Isto é uma excepção ao que ocorre em todos os outros domínios da espinal medula, onde apenas um ligando da via Notch é suficiente para regular a neurogénese. Possivelmente o ligando *Dll1* está envolvido na activação da via Notch e consequentemente na manutenção de progenitores, enquanto o ligando *Dll4* está envolvido na especificação dos interneurónios.

Como é que a expressão de *Dll4* é regulada no domínio V2 da espinal medula e como é que a especificação de interneurónios é controlada neste domínio são os principais temas investigados nesta tese. Os organismos modelo utilizados para estudar estas questões foram o embrião de ratinho e de galinha.

Recorrendo à técnica de hibridação *in-situ* comecei por mapear a expressão de *LFng* (lunatic fringe) e *Hes6* de modo a verificar de que modo a sinalização Notch via *Dll1* ou *Dll4* poderiam ser diferentes. Verifiquei que *LFng* é expresso em células que expressem *Dll4* mas não em células que expressem *Scl* (marcador de V2b). *Hes6* é expresso em células que expressem *Dll1* ou *Dll4* mas não em células Scl^{+}, o que parece sugerir que, de facto, a sinalização Notch através destes dois ligandos é diferente.

De modo a verificar como o *Dll4* é regulado no domínio V2, foquei-me em três proteínas proneurais (MASH1, NGN1 e NGN2) que são expressas em progenitores do V2 e que, segundo abordagens bioinformáticas anteriores, são boas candidatas a regular a sua expressão.

Primeiro foram caracterizadas as células que expressam *Dll4* e cada um dos genes proneurais através de hibridações *in-situ* duplas. As células *Dll4* podem co-expressar os três genes proneurais testados mas a diferentes níveis.

Para testar se estas proteínas podem regular a expressão de *Dll4*, plasmídeos contendo os genes que codificam cada uma destas proteínas foram injectados (sozinhos ou em combinação) na espinal medula de embriões de galinha no estádio HH16-17 pela electroporação *in ovo,* de modo a sobre-expressar estas proteínas. Todos os embriões foram testados em termos de expressão de *Dll4* e especificação de interneurónios.

No que diz respeito à expressão de *Dll4*, a electroporação de *Mash1* ou de *Ngn1* leva a uma repressão de *Dll4* no interior do domínio V2, mas apenas *Ngn1* é capaz de induzir ectopicamente a expressão de *Dll4*. A sobreexpressão de NGN2, não altera a expressão endógena de *Dll4*, indicando que esta proteína proneural não será um factor importante no controlo da expressão deste gene. A electroporação dupla de *Mash1* com *Ngn1* ou de *Mash1* com *Ngn2* reprime também a expressão de *Dll4* no domínio V2 mas leva à sua expressão ectópica noutros domínios ventrais da espinal medula. A sobreexpressão de NGN1 e NGN2 não parece afectar a expressão de *Dll4* na espinal medula.

Visto que as proteínas proneurais controlam a especificação de neurónios durante o desenvolvimento de Sistema Nervoso Central, foi analisado se a sobreexpressão destas proteínas afectaria o número de interneurónios V2a. Após a sobreexpressão de MASH1, uma diminuição no número de interneurónios V2a foi observada, indicando que esta reprime, directa ou indirectamente, a produção destes interneurónios. Contrariamente e após a sobreexpressão de NGN1 ou NGN2, o número de interneurónios V2a aumenta, o que sugere que estas proteínas promovem directamente a diferenciação de interneurónios V2a. A sobreexpressão em diferentes combinações também leva a um aumento de interneurónios V2a no domínio V2.

Foi ainda analisado se o número de progenitores de V2a através da análise de BHLHB5 e LIM3. Verifiquei que MASH1 e NGN1 reprimem o número destes progenitores enquanto Ngn2 não os afecta.

Por hibridação *in situ* foi verificado se o número de interneurónios V2b também seria afectado e verifiquei que em todas as condições o número de V2b sofre uma diminuição, indicando que todas as proteínas proneurais reprimem, directa ou indirectamente a produção destes interneurónios.

Além disto foi analisado o que acontecia à especificação DV da espinal medula aquando da sobreexpressão das proteínas proneurais. Para isso foi analisado o domínio MN que mantem interacções antagónicas com o domínio V2, através da expressão de OLIG2. Verificou-se que o número de células $OLIG2^{+}$ aumentou quando MASH1 é o sobre-expressa ou quando as combinações de proneurais são sobre-expressas.

Assim verificou-sei que MASH1 e NGN1 regulam a expressão de *Dll4* e, consequentemente a especificação de interneurónios enquanto NGN2 não parece regular a expressão de *Dll4*, afectando apenas a especificação de interneurónios.

Este estudo apresenta novas evidências sobre a função que diferentes proteínas proneurais têm na regulação da expressão de *Dll4* e na especificação de interneurónios no domínio V2.

Palavras-chave: Neurogénese, Espinal medula, Domínio V2, Sinalização Notch, Delta-like 4, Proteínas proneurais.

ABBREVIATIONS

AP – Anterior-Posterior
AP – Alkaline Phosphatase
ASC - Achaete-Scute
ATO - Atonal
bHLH – basic Helix-Loop-Helix
BMP - Bone Morphogenetic Protein
CNS – Central Nervous System
CSL - CBF1, Suppressor of Hairless, Lag-1
DIG - Digoxigenin
Dll – *Delta-like*
DNA – Deoxyribonucleic acid
DSL - Delta/Serrate/Lag-2
DV – Dorsal-Ventral
E – Embryonic day
EDTA – Ethylenediamine tetraacetic acid
FBS – Fetal Bovine Serum
FGF – Fibroblast Growth Factor
FP – Floor Plate
HES - Hairy and Enhancer of Split homologues
HH stage – Hamburger and Hamilton stage
IN – Interneuron
IVZ – Inner Ventricular Zone
LB – Luria Bertani bacterial médium
MN – Motor Neuron
MZ – Mantle Zone
NC – Notochord
NGN – Neurogenin
NECD – Notch Extracellular Domain
NICD – Notch Intracellular Domain
NLS – Nuclear localization signal
o/n – Overnight
PCR – Polymerase Chain Reaction
PFA – Paraformaldehyde
POD – Peroxidase
RA – Retinoic Acid
RBPJ – Recombination signal binding protein for immunoglobulin kappa J
RNA – Ribonucleic acid
RP – Roof plate
RT – Room Temperature
SD – standard deviation
Shh - Sonic Hedgehog
SOB – Super Optimal Broth
TSA – Tyramide Signal Amplification
VEGF – Vascular endothelial growth factor
VZ – ventricular zone

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CHAPTER I

Introduction

Chapter I – Introduction

The vertebrate nervous system contains a great diversity of neurons and glial cells interconnected to form highly complex neural networks. These cells are generated in the embryonic neural tube at specific time and position in order to accurately interact with each other and to assemble into a functional network.

I. 1. VERTEBRATE NEUROGENESIS AND PATTERNING

For the central nervous system (CNS) to be functionally assembled not only the balance between progenitors and differentiating neurons must be controlled but also the position where specific neurons are generated must be tightly regulated.

After neural tube formation, neural progenitors acquire different fates according to their relative positions along the anterior-posterior (AP) and dorsal-ventral (DV) axes of the embryo. The neural tube will originate the brain anteriorly (from which the forebrain, midbrain and hindbrain will be formed) and the spinal cord posteriorly [1].

Neural progenitors of the neural tube normally develop anterior identity and differentiate into forebrain neurons. While some neural progenitors along the AP axis of the neural tube need to be kept in an undifferentiated state in order to gradually acquire different identities, others will differentiate into midbrain, hindbrain and spinal cord neurons. The anterior secretion of retinoic acid (RA), which promotes neuronal differentiation, and the posterior secretion of FGF, which represses neuronal differentiation, by surrounding mesodermal tissues, is responsible for the generation of the CNS in a rostral-to-caudal sequence, allowing progenitors to gradually differentiate in the correct moment and position (Figure 1) [2, 3].

Along the dorsal-ventral axis of the developing spinal cord, neural progenitors can be subdivided into eleven molecularly distinct domains (six dorsal and five ventral)[4]. Within each of these domains, populations of progenitor cells are defined by distinct combinations of transcription factors, induced by different levels of morphogens, namely by the bone morphogenetic proteins (BMPs) and WNT's produced dorsally (either by roof plate or dorsal epidermis), and Sonic hedgehog (Shh), secreted ventrally by notochord and floor plate (Figure 1) [4, 5, 6, 7]. These morphogens confer specific positional identities, activating region-specific differentiation programmes and, therefore, specifying the identity of neurons that derive from individual progenitor populations [8, 9, 10].

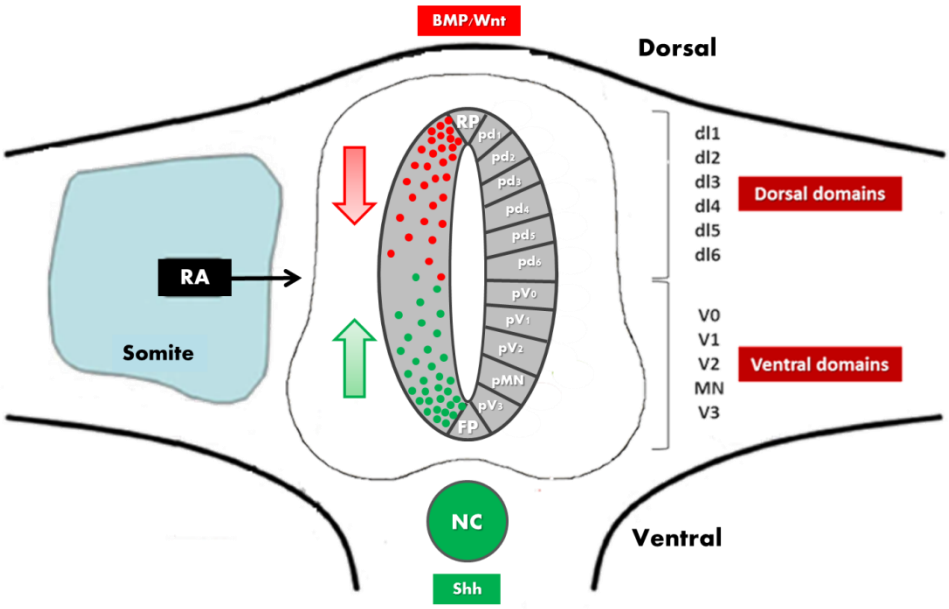

Figure 1 - **Diagram of a transverse section of the spinal cord**. Different populations of neural progenitors acquire different fates according to their position along the dorsal-ventral axis. The patterning is established by gradients of Shh, secreted by the floor plate (FP) and the notochord (NC), and Wnts and BMPs, produced by the roof plates (RP) and the dorsal epidermis. The Retinoic Acid (RA) produced by the adjacent somites is also involved in the patterning (AP and DV) of the spinal cord.

I.2. NOTCH SIGNALLING IN VERTEBRATE NEUROGENESIS

Notch signalling is one of the most conserved pathways in the regulation of metazoan development. It is involved in different processes like body segmentation (e.g. segmentation clock), skeletal development, embryonic haematopoiesis and neural development, including neuronal differentiation and glial determination controlling several biological functions such as apoptosis, cell proliferation and cell fate decisions, (Reviewed in [11]).

The Notch pathway is a cell-cell communication system that results from the interaction of surface proteins: a Notch receptor and its ligand. The Notch receptors are a type I transmembrane heterodimeric proteins present at the plasma membrane. These receptors are composed by an ectodomain called NECD (Notch Extracellular Domain) and a membrane bound intracellular domain, which are conserved between species [12].

Notch receptors are activated by transmembrane ligands of the DSL family (Delta and Serrate from *Drosophila* and Lag-2 from *C.elegans*). The DSL ligands are type I transmembrane proteins with an extracellular domain that is conserved across species and an intracellular domain that present a small conservation even between different types of ligands [12].

Despite being a highly conserved pathway, different organisms have different receptors and ligands, for example, in Drosophila, there is only one Notch receptor and

two ligands (*Delta* and *Serrate*), while in chick two Notch receptors (*Notch1* and *Notch2*) and four ligands (*Delta1, Delta4, Serrate1* and *Serrate2*) have been described. In mammals there are four receptors (*Notch1, Notch2, Notch3* and *Notch4*) and five ligands (*Delta-like1* (*Dll1*), *Delta-like3* (*Dll3*), *Delta-like4* (*Dll4*), *Jagged1* (*Jag1*) and *Jagged2* (*Jag2*)) [13, 14]. This thesis will focus on *Dll4*.

In this pathway a cell expressing the Notch ligand (signal-sending cell), either *Delta* or *Serrate*, signals to the neighbour cell, which expresses a Notch receptor (signal-receiving cell). Notch receptors are formed in the trans-Golgi as a result of a proteolytic cleavage at the site S1 (Figure 2). Whenever a ligand protein binds to the extracellular domain of the Notch receptor, this one undergoes successive proteolytic cleavages. The first cleavage occurs at the extracellular S2 site and it is mediated by extracellular proteases of the ADAM/TACE family. The S2 cleaved form of Notch receptor is then processed at the endomembrane S3 site by the γ-secretase. After the proteolytic cleavages there is a release of the NICD (Notch Intracellular Domain) that is translocated to the nucleus where it associates with a DNA-binding protein called CSL (in human, CBF1; in *Drosophila*, Suppressor of Hairless; and in *C.elegans*, Lag-1) and to Mastermind co-activator, forming a nuclear complex which recruits other factors to regulate the expression of its target genes (Figure 2). In the absence of NICD, the transcription factor CSL is part of a transcriptional repressor complex that represses genes containing promoters with CSL-binding sites, whereas in the presence of NICD it activates the transcription of the same genes (Figure 2) [11, 15,16].

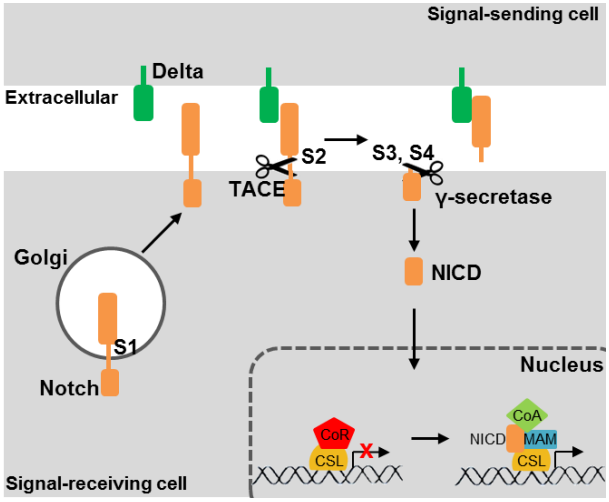

Figure 2 – Notch signaling pathway. Whenever a ligand (*Delta*) expressed in the signal-sending cell binds to the extracellular domain of Notch receptor in the signal-receiving cell, it leads to a conformational change in the receptor and, as a consequence three successive proteolytic cleavages occur (at sites S2, S3 and S4) mediated by TACE/ADAM and γ-secretase, respectively. There is a release of NICD that is translocated to the nucleus where it associates with CSL and Mastermind, forming a complex that recruits other factors and activate the transcription of target genes.

I.2.1. Notch Targets

There are several CSL binding sites throughout the genome but the best described Notch transcriptional targets are genes encoding bHLH (basic Helix-loop-helix) transcriptional repressors. These repressors include the *Enhancer of Split (E(spl)) complex* in *Drosophila* and the *Hairy and Enhancer of Split* homologues (HES)

or *Hes*-related (*Hesr/Hey*) families of proteins in vertebrates. All these proteins are bHLH-Orange (bHLH-O) as they contain a conserved amino acid sequence – Orange domain – located C-terminal to the bHLH domain [17, 18, 19].

The HLH domain allows homo- and heterodimerization of these bHLH-O proteins, which can then exert their function by DNA-binding and transcriptional repression. These proteins can bind DNA in consensus sequences called E-boxes (CANNTG) or N-boxes (CACNAG) by their basic domain. After DNA binding, the C-terminal motif WRPW recruits co-repressor factors, like Groucho, and this leads to the transcriptional repression of target genes, like proneural genes. Moreover, bHLH-O proteins can repress transcription by directly interact and form heterodimers with bHLH activator proteins, through the HLH domain, and this prevents binding of the activators to DNA, thereby stopping their transcriptional activity [18, 20].

I.2.2. Notch Signalling Pathway during Vertebrate CNS Development

During CNS development, the Notch pathway has a critical role in neuronal differentiation and also in glial determination through different mechanisms. Regarding the neural tube, Notch receptors are expressed in uncommitted neural progenitor cells, which are arranged in a polarized neuroepithelium in the ventricular zone (VZ) [21].

During vertebrate neurogenesis, Notch signalling has been shown to mediate cell fate decisions by maintaining neural progenitor identity, while supressing neuronal differentiation. In the vertebrate neural tube, the walls are composed by neuroepithelial cells that can differentiate into neurons. These neuroepithelial cells are polarized, with the basal region in contact with the basal lamina at the periphery of the tube, whereas the apical end is next to the lumen of the tube. Cells are in close contact to one another in the apical region by a variety of specialized junctions (e.g. tight junctions) [21]. The neural progenitors are bound at the apical and basal surfaces of the neuroepithelium but their nuclei migrate along the axis of the cell accordingly to the cell cycle phase: M-phase nuclei are at the apical side while cells in S-phase have their nuclei close to the basal surface. During G1 and G2 phases, the nuclei migrate between these two opposing positions, in a movement known as interkinetic nuclear migration. However, after division, each of the daughter cells either repeats or exits the cell cycle. When the cell exits the cell cycle, it loses the apical attachment and migrates out of the ventricular zone to the mantle zone (MZ) where it starts to differentiate [21, 22].

I.2.2.1. Maintenance of neural progenitors through lateral inhibition

Notch signalling can act to promote cell diversity through a process called lateral inhibition. In this process, a group of cells with a similar developmental potential

can give rise to different cell types and it ensures that two interacting cells do not acquire the same fate [9, 23].

Regarding the maintenance of neural progenitors, two neighbouring cells signal to each other and one starts the process of neuronal differentiation while the other is kept as a progenitor. The decision of becoming a neuron or remaining as a neural progenitor is controlled by the balance between two different sets of transcription factors: proneural bHLH proteins, which instruct progenitors into neuronal differentiation, and HES proteins, which repress neuronal differentiation and therefore maintain cells as progenitors. Nevertheless, due to lateral inhibition, the decision of each neural progenitor is influenced by the fates of the neighbour cells. Neural progenitors in the VZ of the neural tube express proneural proteins, but stochastic variations in gene expression causes one cell to express higher levels of proneural genes and therefore start the differentiation process [24, 25]. It is known that proneural proteins positively regulate the expression of Notch ligands, so the expression of these ligands will be higher in the differentiating cell (Figure 3) [26]. By lateral inhibition, this cell will then signal to neighbouring cells expressing Notch receptors, leading to an increase in HES expression, which will repress proneural genes and prevent premature differentiation of the cells into neurons (Figure 3) [9].

Lateral inhibition mediated by Notch signalling provides a feedback mechanism

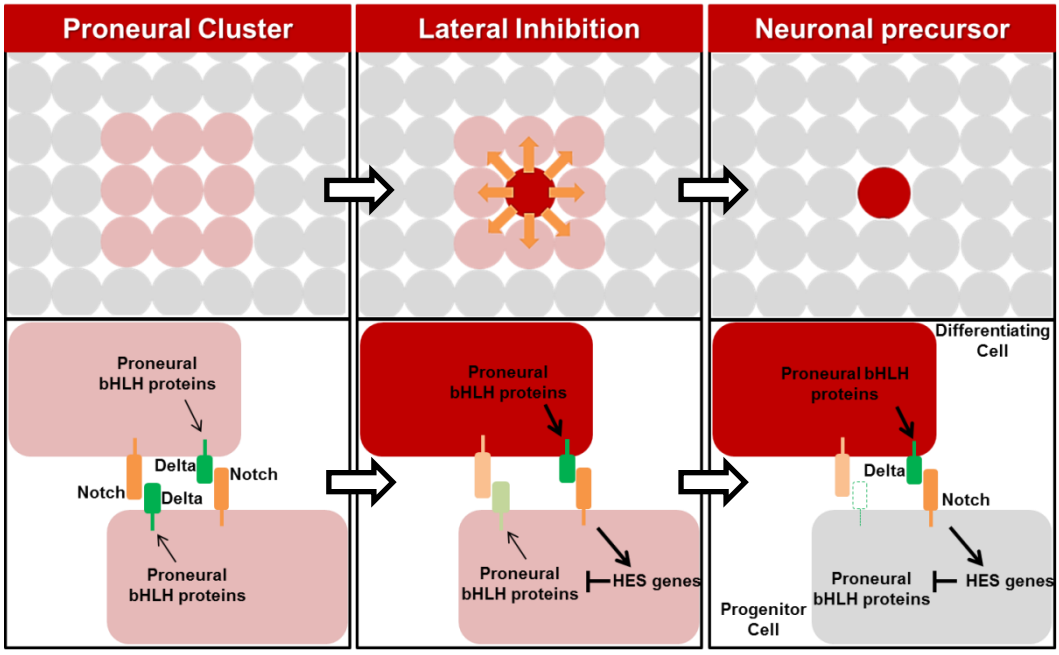

Figure 3 – Lateral Inhibition mechanism. Notch signalling amplifies small or weak differences within roughly equivalent populations of cells. Cells in the proneural cluster express proneural proteins. One cell expresses higher levels of proneural proteins and start differentiating. This will regulate positively the expression of *Delta*, which by lateral inhibition causes this cell to signal to its neighbours (*Notch*$^{+}$).Notch signalling is activated in the surrounding cells, increasing the expression of HES genes, which repress proneural proteins. These cells will remain as progenitor cells, while the other cell will differentiate. Adapted from Gilbert, 2006.

to control the production of neurons [23]. Also it maintains a pool of neural progenitors throughout neurogenesis, which allow these cells to be exposed to different environmental cues and to differentiate into different neurons during development. Furthermore, as neurons and glia cells are produced from the same progenitor pool, the maintenance of these cells by Notch signalling allows the later production of glia cells, after neurogenesis [11].

I.2.2.2. Cell fate specification

Notch signalling is also important in the specification of neuronal fates. Several studies indicate a role of Notch signalling not only in progenitor maintenance but also as an instructive cue to the production of glial cells [11, 27, 28].

In the vertebrate CNS, neurons are generated before glial cells. The shift from neurogenesis to gliogenesis involves a decrease in gene expression required for neurogenesis and an increase in expression of genes required to gliogenesis [11]. Although Notch signalling might induce the formation of glial cells, like astrocytes, it may also contribute to inhibition of the oligodendrocyte fate [29].

Notch signalling is also proposed to regulated cell-type specification in ear, retina and spinal cord development [28, 30]. In the retina, the differentiating retinal neurons strongly express the Notch ligand, *Dll4*, and it is known that Notch-Dll4 signalling is involved in cell-fate choice steps during retinogenesis [14]. The next section of this thesis will focus on the spinal cord case.

I.3. SPINAL CORD NEUROGENESIS – THE V2 DOMAIN

In the developing spinal cord, along the DV axis, there are eleven molecularly distinct domains in the spinal cord: six dorsal and five ventral (Figure 1) and each one of them express at least one Notch ligand. The most widespread ligand is *Dll1*, which is expressed in the majority of dorsal and ventral domains, being only absent in dl6 and V1 domains where *Jag1* expression occurs [13]. However, in the V2 domain, a second ligand is expressed, *Dll4*, together with *Dll1* [13, 14].

This is an exception to what seems to be a general rule exhibited in other domains of the developing spinal cord, where only one Notch ligand (*Dll1* or *Jagged1*) is sufficient to regulate neurogenesis [13, 14]. Remarkably, in the V2 domain, *Dll4* is only expressed in a small number of cells that are starting neuronal differentiation [31]. Another unique feature of the V2 domain is that the p2 cells (progenitor cells from the V2 domain) co-express different proneural bHLH proteins (MASH1, NGN1 and NGN2), suggesting that combinations of these transcription factors may regulate the expression

of *Dll1* and *Dll4* in this domain [32]. The V2 domain is also particular as it gives rise to three functionally distinct classes of interneurons: V2a, V2b and V2c [33], all generated from a pool of molecularly similar progenitors with a specific molecular identity (e.g. *Nkx6.1, Irx3* and *Pax6*) [5, 34, 35]. V2a and V2b interneurons are specified over the same time window but they have different physiological functions: V2a are excitatory glutamatergic INs, V2b are inhibitory GABAergic INs and V2c function is still unknown [33, 36].

Notch-Delta interactions have been suggested to play an important role in the initiation of a binary cell fate choice in immature V2 interneurons [30, 37, 38]. Within the VZ of the V2 domain, cells that start the differentiation process express high levels of proneural proteins, and therefore high levels of *Dll1*, activating Notch in the neighbouring cells (signal-receiving cells), keeping them as progenitors (Figure 4) [32, 33]. Some V2 cells that are committed to differentiation will express high levels of *Dll4* and will differentiate into V2a INs (Ramos, C. et al, unpublished). These *Dll4*$^{+}$ signal-sending cells have been shown to downregulate *Gata2* [39], while maintaining the expression of *Lim3* (*LIM homeobox 3*) [38, 40, 41]. These cells will then downregulate *Lim3* and activate the expression of *Bhlhb5* and *Chx10*, adopting the V2a cell fate [37, 42]. The remaining cells committing to differentiation, do not express *Dll4* and, will downregulate *Lim3* [40], while maintaining the expression of *Gata2* [39], and activate the expression of *Scl* (*Stem cell leukaemia*) and *Gata3* to adopt V2b cell fate (Figure 4) [37, 42, 43].

Even though it is known that Notch signalling is crucial in V2 cell fate specification, how *Dll1* and *Dll4* ligands mediate this process is still under study. Previous analyses of *Dll1*-mutant mice (conditional knock-out) reveal that both V2a and V2b INs are generated, although in different numbers (increased number of V2a INs), when compared to control littermates [14, 30, 42]. In *Notch1* mutants all V2 progenitors acquire V2a fate, at the expense of the V2b fate [42]. Furthermore, overexpression of DLL4 in chick spinal cord increases the number of V2b INs at the expense of V2a INs, whereas overexpression of DLL1 doesn't affect significantly the number of V2a vs. V2b[30]. These results suggest that Notch signalling is required for the generation of V2b

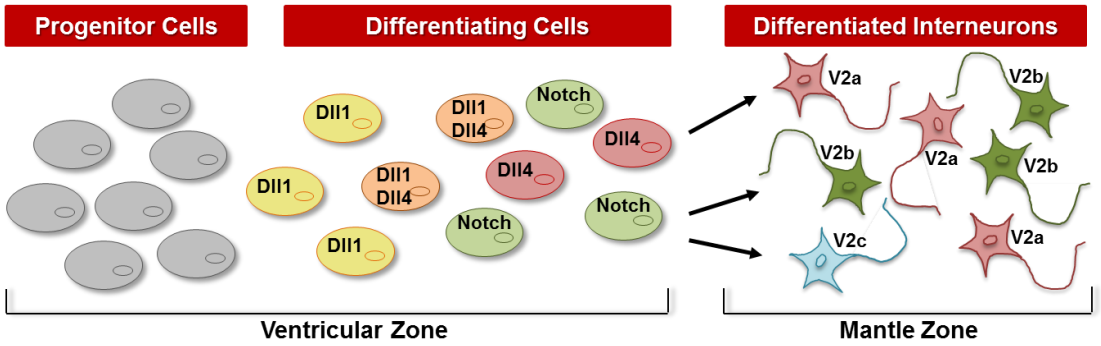

Figure 4 – V2 domain of the developing spinal cord. Cells starting the differentiation process express high levels of proneural proteins and, therefore, high levels of *Dll1*. These cells activate Notch signalling and keep the surrounding cells as progenitors. Some of the *Dll1*-expressing cells will express *Dll4* and will differentiate into V2a INs while other V2 cells receive signals through the Notch receptor and differentiate into V2b and V2c INs.

INs and that *Dll1* is not the key ligand for the V2a-V2b binary switch, a decision that might be controlled by *Dll4*-mediated Notch signalling [13, 14].

I.4. REGULATORS OF *Dll* EXPRESSION AND CELL TYPE SPECIFICATION

I.4.1. bHLH Proneural Proteins

Proneural transcription factors contain a Helix-Loop-Helix (HLH) domain that allows these proteins to dimerize and, subsequently, to bind DNA through their basic domain. The proneural proteins were first identified in *Drosophila* and were divided into two families: the Atonal (ATO) and Achaete-scute (ASC) families (Reviewed in [9]). The vertebrate Achaete-scute (ASC) family includes Ash1 (e.g. MASH1 in mouse, CASH1 in chick and ZASH1 in zebrafish) and other genes that are specific for vertebrate classes (MASH2 in mammals, XASH3 in *Xenopus* and CASH4 in chick) [9]. The number of vertebrate proteins related to *Drosophila* ATO family is larger, but only two of them (MATH1 and MATH5 in mouse) have a bHLH domain similar enough to that of ATO to be considered as orthologues (reviewed in [9]). Other vertebrate ATO-related proteins can be grouped into distinct families, e.g., the Neurogenin (NGN) family, the NeuroD family and the Olig family [9].

Some of the above mentioned proneural proteins are expressed in the V2 domain, specifically MASH1, NGN1 and NGN2, making them good candidates to regulate neurogenesis in this domain [32]. Previous data suggest that *Mash1* is expressed in V2 progenitors but how it is involved in interneuron specification is still controversial: Parras et al. reported a decrease in V2a interneurons in *Mash1*-mutant mice while the opposite result is reported by Li et al. *Ngn1* and *Ngn2* are also expressed in V2 cells but how their expression might influence V2 cell fate is still under study. In fact, in zebrafish, expression of *DeltaD* (Notch ligand) in the brain and in the spinal cord has been shown to be regulated by NGN and ZASH1 [44] and, in mouse spinal cord and brain, MASH1 and NGN1/2 have been reported to directly regulated *Dll1* expression [26]. The regulation of *Dll4* expression during spinal cord development is one of the aims of this thesis.

I.4.2. Regulation of *Dll4* Expression

Dll4 might have an important role for interneuron specification in the V2 domain. Therefore, *Dll4* expression must be tightly regulated for the correct number and type of V2 INs to be produced.

Regulation of *Dll4* expression has been extensively studied in endothelial cells, since Notch signalling mediated by *Dll4* has been implicated in vascular growth [31].

Here, vascular endothelial growth factor (VEGF) induces *Dll4* expression as part of a negative regulatory loop, in which *Dll4* act as an inhibitor of vascular sprouting. Also *Dll4* is likely to be involved in the modulation of diverse forms of pathological angiogenesis, like in tumour angiogenesis. The blockade of *Dll4* was found to delay tumour growth by enhancing the abnormal vascular sprouting, characteristic of tumour angiogenesis [45]. Arterial specification is regulated by the combinatorial function of Notch signalling and SoxF transcription factors, via direct transcriptional gene activation. Arterial *Dll4* expression requires the direct binding of both RBPJ (CSL)/NICD and SoxF transcription factors and this occurs downstream of the action of VEGF. The combinatorial role may contribute to more precise spatial and temporal control of gene expression within the differentiating vasculature [46]. In endothelial cells, the coordinated activation of Notch signalling produces a wave of *Dll4* expression. However in the V2 domain of the developing spinal cord, *Dll4* is expressed in a salt-and-pepper pattern, with cells expressing high levels of *Dll4* surrounded by cells that express low levels of this ligand. Hence, the mechanism behind *Dll4* expression in the V2 domain is most likely different from the mechanism controlling Dll4 expression in endothelial cells.

Due to their expression in the V2 domain, good candidates to regulate Dll4 expression are the proneural proteins MASH1, NGN1 and NGN2.

Published work demonstrates that, in the chick developing spinal cord, the proneural protein CASH1 is involved in the regulation of *Dll4* expression [30]. In mouse, *Dll4* expression has been suggested to be regulated by both MASH1 and FoxN4, a winged-helix forkhead protein, expressed in some V2 progenitor cells [42, 47, 48].

I.5. AIMS

The aim of this thesis is to unravel: i) how *Dll4* expression is regulated in the V2 domain; ii) how is *Dll4* biased expression involved in V2 cell type specification. Particularly, I investigated how the asymmetric expression of *Dll4* emerges from initially identical progenitor cells by testing whether there is an induction of *Dll4* in some V2 cells or if is there a repression of *Dll4* in some cells in the V2 domain, using in *ovo* electroporation of regulator candidates. The chicken embryo was used as a model organism in this approach due to its accessibility and easy handling.

Furthermore I characterized *Dll4*-expressing cells and unraveled if there is a difference in *Dll1* and *Dll4*-mediated Notch signaling in the V2 domain, using mouse embryo as a model due to its genetic tools availability.

CHAPTER II

Materials and Methods

CHAPTER II – MATERIALS AND METHODS

II.1. MOLECULAR BIOLOGY TECHNIQUES

II. 1.1. Plasmid Transformation of chemically competent *E.coli* bacteria

For plasmid DNA transformation, 100µL of competent *E. coli* bacteria were used. Competent bacteria were first thawed on ice and then incubated with plasmid for 20 min on ice. The mixture was heat-shocked for 45 seconds in a water bath at 42°C and then incubated on ice for 2 min. After adding 900µL of Super Optimal Broth (SOB) medium supplemented with 10mM $MgCl_2$ and 10mM $MgSO_4$, bacteria were incubated at 37°C for 1 hour, shaking. 100µL of the mixture were plated on the appropriate selective LB agar media and incubated at 37°C o/n.

II. 1.2. DNA Constructs – Plasmid DNA purification

Plasmid constructs were stored at -80ºC as bacterial stocks (400µl of an o/n grown bacterial culture carrying the plasmids and 400µl of LB containing 30% glycerol). For large scale preparation of plasmid DNA (200-400µg), 100mL of the selective LB medium was inoculated with 1mL of plasmid bacterial culture (previously grown o/n). Bacteria were grown at 37ºC and processed using *Genopure Plasmid Midi Kit* (Roche), according to the given instructions.

II. 1.3. DNA Quantification

DNA concentration was determined by spectrophotometry using the NanoDrop spectrophotometer (Thermo Scientific) – see details in Annex A.

II. 1.4. Analytical digestions

Analytical digestions were performed to confirm gene size and identity (by fragment profile analysis). DNA was digested for 1-2h using 5-10U of commercially available enzymes and respective buffers. Restriction analysis was then performed in 1% agarose gel.

II.2. CHICK EMBRYO MANIPULATION

II.2.1. Chicken embryos

Fertilized chicken eggs were stored for a maximum of one week at 15ºC and incubated ate 37ºC in a humidified incubator (SMA 60) until the required developmental stages.

II.2.2. *In ovo* chick embryo electroporation

Embryos were injected with plasmid DNA using capillary needles made from borosilicate glass capillaries. The plasmid DNA was injected in spinal cord of chicken embryos at HH16-18 using the Inject+Matic (Genève®) injector (1 µg/µl). Each plasmid DNA was co-injected and co-electroporated with mCherryNLS@pCAGGS so that the electroporated cells can be visualized and act as a positive control for electroporation efficiency. Each plasmid DNA was injected with filtrated Fast green in order to visualize the injected solution. Platinum electrodes were placed 4mm apart of each other and parallel to the neural tube and the embryos were pulsed 5 times (25V/50 ms) using the Electro Square Porator™ ECM830 (BTX). Embryos were incubated again and after 36 hours were harvested and fixed in a 4% paraformaldehyde (PFA) in PBS solution at 4ºC o/n.

II.2.3. Tissue embedding and preparation of cryostat sections

After fixation, embryos were washed twice in PBS and then transferred to a 15% and then 30% sucrose in PBS solution for cryoprotection. The embryos were embedded in a solution containing 7.5% gelatin and 15% sucrose in PBS and frozen in cold isopenthane (-75ºC). The frozen embryos were stored at -80ºC until sectioned in a cryostat (Leica CM 3050). Embryonic tissue was sectioned (12 µm) and collected on Superfrost® slides.

II.3. MOUSE EMBRYO MANIPULATION

Mouse embryos, both wild-type and mutant (single and double mutants for *Dll1* and *Dll4*) were collected at embryonic day 11.5. For the generation of these embryos, males with Cre-recombinase under regulation of Nestin promoter (specifically expressed in neural cells) and floxed for *Dll1* and/or *Dll4* were crossed with floxed females for *Dll1* and/or *Dll4*. Crosses were set and vaginal plugs checked routinely to confirm pregnancy and, when confirmed, E11.5 embryos and the corresponding yolk sacs were collected. Pregnant females were sacrificed and a caesarean section performed to collect the embryos. All animals were fed freely and housed in SPF facilities. Animal experiments were approved by the Animal Ethics Committee of Instituto de Medicina Molecular and according to National Regulations.

After embryo collection, the yolk sacs were denatured and digested and the isolated DNA was used for genotyping with PCR for the presence of Cre in a first step and then for *Dll1* and *Dll4*, followed by gel electrophoresis. Embryos without Cre were treated and used as wild-types control embryos. Tissue embedding and preparation of cryostat sections was the same as for chick embryos (previously described).

II.4. *IN SITU* HYBRIDIZATION

II.4.1. Antisense RNA probe synthesis

Several RNA antisense probes were used to perform *in situ* hybridization on whole-mount or cryostat sections of both mouse and chick embryos. Digoxigenin- (DIG) and Fluorescein- (FLUO) labeled RNA antisense probes were synthesized *in vitro* by T3 or T7 RNA polymerase, from several plasmid templates (see Table 2 – Annex A).

II.4.1.1. DNA template preparation

Different Plasmid DNA constructs (10µg) were linearized using 100U of the specific restriction enzyme in a final volume of 100 µl for 1 hour at 37ºC. After checking complete digestion by running 1 µl in an agarose gel 1%, DNA template was subjected to column purification and cleanup using *Wizard Plus SV Gel and PCR Clean-up System* (Promega). DNA was quantified (as previously described).

II.4.1.2. Probe synthesis

Antisense probes were produced using 1µg of linearized plasmid DNA and 20U of RNA polymerase (T3 or T7) with 30mM DTT, 1x DIG-NTP mix (1mM ATP, CTG, GTP, 0.65mM UTP and 0.35mM DIG-UTP), 40U of RNase inhibitors (Roche), 1x Transcription Buffer (Stratagene) and RNase-free water in a final volume of 25µl. After 3 hours of incubation at 37ºC, the sample was precipitated by adding 20.5µl of RNase-free water, 2µl 0.5M EDTA, 2.5µl of 8M LiCl, 150µl of 100% ethanol and 1µl of glycogen and incubated o/n at -20ºC. The samples were centrifuged and the supernatant was discarded, RNA precipitate was washed with 70% ice-cold ethanol and then ressuspended in 100µl of 10mM EDTA and stored at -20ºC. To check for the quality of the probe, 2µl were mixed with RNA loading buffer containing formamide and, after a denaturation step of 5 min at 70ºC, run in agarose gel along with a probe of known concentration.

II.4.2. Fluorescent *In situ* hybridization

Fluorescent *In situ* hybridization on cryostat section was done by hybridizing DIG- or Fluo-labelled antisense RNA probes o/n at 68ºC in a humidified chamber wetted in 1x salts/50% formamide. Probes were diluted (1:100) in hybridization buffer and denatured at 70ºC for 10 min. After o/n hybridization, sections were washed for 10 min with pre-warmed washing solution at 68ºC to remove coverslips and then washed twice with the same solution and temperature for 15 min. Sections were washed three times for 15 min with TBST at RT and blocked with a solution of 2% blocking reagent and 20% heat inactivated sheep serum in TBST for more than 1 hour at RT in a

humidified chamber. Section were then incubated with antibodies anti-DIG AP or anti-FLUO AP (Roche, 1:2000) o/n at 4ºC. After o/n incubation, sections were washed three times with TBST for 10 min and then twice with 0.1M Tris for 10 min. The staining reaction for AP (alkaline phosphatase) was performed using Fast Red (Roche) for 1-3h at 37ºC. After the development of the probe, sections were washed twice in PBS and incubated with the antibody that recognizes the second probe (anti-FLUO-POD, anti-DIG-POD or anti-DNP-POD, Roche, 1:500). After 1 hour of incubation in a humidified chamber at RT, sections were washed with 1x TNT three times for 10 min and the staining reaction for POD (peroxidase) was performed using Tyramide Signal Amplification (1:50, TSA) for 20 min at RT. Sections were then washed four times with 1x TNT for 5 min, counterstained with DAPI and mounted with Mowiol mounting medium.

II.5. IMMUNOFLUORESCENCE

Gelatin was removed from selected regions with pre-warmed PBS1x at 37ºC, sections were treated with H_2O_2 in methanol for 30 min at RT to reduce background by blocking endogenous peroxidases. Sections then, went through a 0.1M Glycine in PBS1x treatment for 10 min at RT to quench paraformaldehyde and stop cross-linking reactions and were permeabilized with 0.5% Triton in PBS1x for 10 min at RT. After this, sections were blocked with 10% Fetal Bovine Serum (FBS) in TBST for 1 hour at RT and then incubated with primary antibodies, previously diluted in 10% FBS in TBST, o/n at 4ºC in a humidified chamber. After primary antibody binding, sections were washed three times with TBST for 10 min at RT and incubated with secondary antibodies (diluted in 10% FBS in TBST) for 1 hour at RT in a humidified chamber. Sections were washed again three times with TBST for 10 min at RT and then counterstained with DAPI for 10 min. After this sections were mounted with Mowiol mounting medium.

The H_2O_2 pre-treatment was not performed on electroporated chick embryos and on mice embryos when fluorescence was to be preserved.

The antibodies used during this project are described in Annex A.

II.6. FIXED TISSUE IMAGING

Images of fixed sections with fluorescence were acquired using Leica DM5000B microscope with a Leica DC350F digital camera. Co-expression analysis was made using Confocal Laser Point-Scanning Microscope – Zeiss LSM 510 META. The acquired images were then treated using Adobe Photoshop software or ImageJ.

II.7. STATISTICAL ANALYSIS

Cell counts were performed using Adobe Photoshop software and were expressed as the number of positive cells for the relevant marker, as a percentage of total DAPI (mean ± SD) from the electroporated or non-electroporated side of the neural tube. Data were analysed using the unpaired *t*-test to compare two data sets (GraphPad Prism v.6.0). Values of $p<0.05$ were considered significant.

Note: The composition of all solutions is described in Annex A, Table 3.

CHAPTER III

Results

CHAPTER III – RESULTS

III.1. NOTCH SIGNALLING IN THE V2 DOMAIN

Within the VZ of the V2 domain, progenitors that start the differentiation process express high levels of *Dll1*, activating Notch in surrounding cells to maintain them as progenitors (these cells express *Hes5* and *LFng*) [17, 49] (Fig. 5A). The *Dll1*-expressing cells can subsequentially express *Dll4* and became prospective (*Bhlhb5*$^{+}$) V2a INs, activating Notch in the surrounding cells that will differentiate into prospective (*Scl*$^{+}$) V2b INs (Fig. 5B). Previous data from our lab showed that *Dll4*-expressing cells will differentiate into V2a INs and activate Notch signalling in neighbouring cells (*Dll4*$^{-}$ and *Scl*$^{+}$) that, in turn, differentiate into V2b INs (Ramos C. et. al unpublished) (Figure 5B).

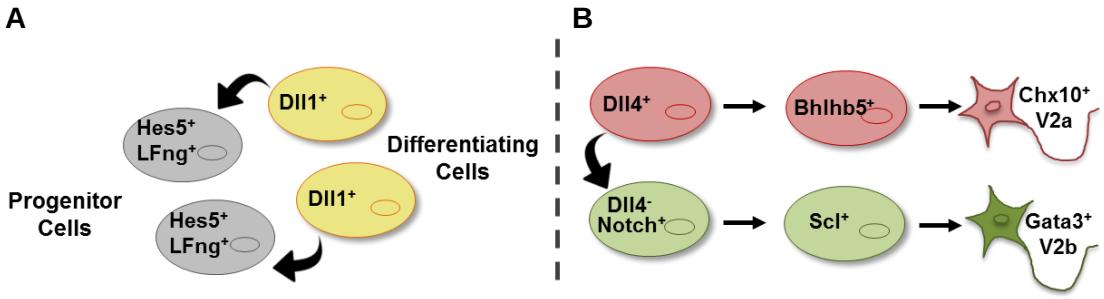

Figure 5 – Cell dynamics inside V2 domain. (A) - Cells that express high levels of Dll1 will keep their neighbours as progenitors.(B) - Differentiating cells can express *Dll4*, upregulate the expression of *Bhlhb5* (prospective V2a IN marker) and became V2a INs (*Chx10*$^{+}$ cell). Their neighbours (*Dll4*$^{-}$ *Notch*$^{+}$ cells) upregulate the expression of *Scl* (prospective V2b marker) and differentiate into V2b INs (*Gata3*$^{+}$ cell).

In the developing spinal cord, the V2 domain is the only one where two Notch ligands are expressed (DLL1 and DLL4), therefore a question arises: Is *Dll1*-mediated Notch signalling different from *Dll4*-mediated Notch signalling? One hypothesis is that, indeed, *Dll1* and *Dll4*-mediated Notch signalling are different due to the presence of *LFng*, a potentiator of Notch signalling [50], in signal-receiving cells. It is known that *Dll1* is keeping the surrounding cells as progenitors, which express *LFng* [49], however concerning *Dll4*-mediated Notch signalling, the expression of *LFng* have not yet been mapped, so our hypothesis is that *LFng* might be involved in *Dll1*-mediated Notch signalling but not in *Dll4*-mediated Notch signalling.

To test this hypothesis, we analysed simultaneously the expression of *LFng, Dll4* and *Scl* in the V2 domain of E11.5 mouse spinal cord. The results show that *LFng* is expressed in the VZ of most spinal cord domains, being only absent in the dl6 and V1 domains, in agreement with previous results (Fig.6 A and B). We did not observe cells expressing both *LFng* and *Scl* (Fig.6 A'-A''') indicating that *LFng* is not expressed in prospective V2b INs. However, *LFng* is co-expressed with *Dll4*$^{+}$ in cells within the V2

progenitor domain (Fig.6 B'-B''' – indicated by arrow), but not in cells outside this region, where only *Dll4* expression can be detected.

These data point out that Notch is activated by *Dll4* in *Scl*$^{+}$ cells without *LFng* potentiator and that *Dll4*-mediated Notch signalling is independent of *LFng* (Fig.5A).

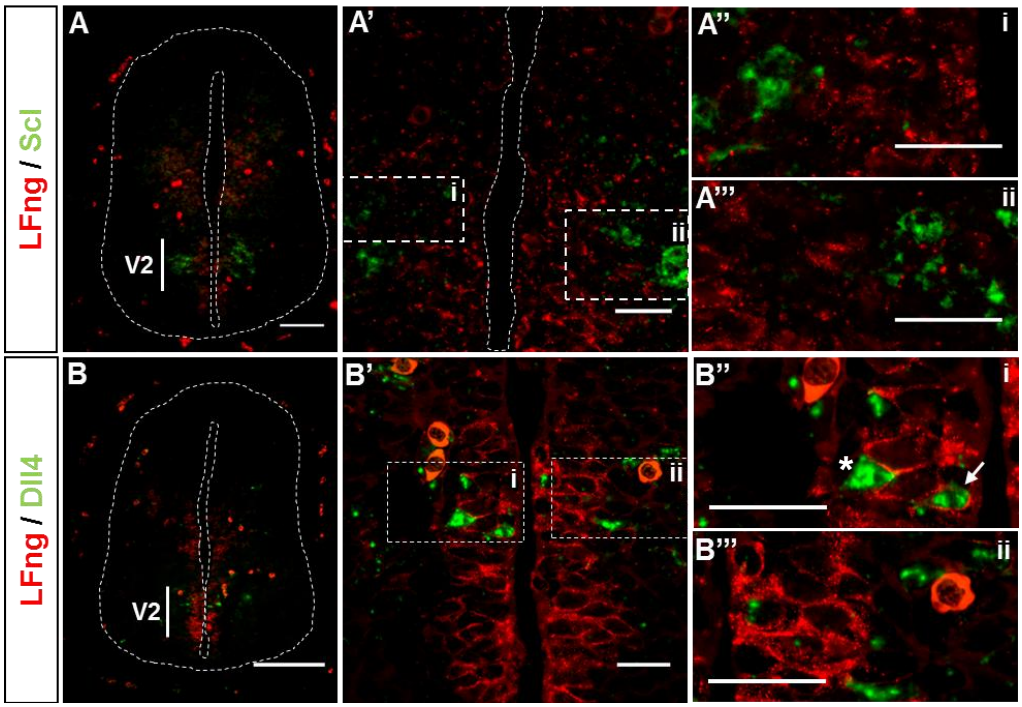

Figure 6 – *LFng* expression in the prospective V2 INs. (A-B) – *LFng* is expressed in the VZ of V2 domain, in progenitor cells. (A') – *LFng* is not coexpressed with prospective V2b marker *Scl*. (A', A'') – magnification of the selected regions from confocal image A'. (B') – *LFng* can be coexpressed with *Dll4*. In the ventricular zone, *Dll4*$^{+}$ cells express also *LFng* (B'' – white arrow) but when they migrate outside this region the co-localization of both mRNAs is no longer detected (B' - indicated by asterisk). (B', B'') – magnification of the selected regions from confocal image B'. Scale bars: A, B - 50µm; A'-A''', B'-B''' - 10µm.

We next analysed the expression of *Hes6*, to try to correlate its expression to the possible differential activity of *Dll1* or *Dll4* ligands. *Hes6* is a downstream target of Notch signalling, expressed in differentiating neurons, in which it functions to release proneural gene expression from the inhibitory effects of downstream targets of Notch signalling (*Hes5*) [51].

To test whether *Hes6* might be activated by *Dll1* and/or *Dll4* we analysed simultaneously the expression of *Hes6, Dll1*, *Dll4* and *Scl* in the V2 domain of E11.5 mouse spinal cords. Our data show that *Hes6* is expressed throughout all domains of the spinal cord (Fig.7 A, B, C), in the VZ. However in the V2 domain, *Hes6* expression is detected in cells located next to the lumen in the inner ventricular zone (IVZ), an exception to what is observed in all the other domains. Cells co-expressing both *Hes6* and *Dll1* can be observed in the V2 domain (Fig.7 A', A'' – indicated by arrows). *Hes6* and *Dll4* were also co-expressed (Fig.7 C'-C''' – indicated by arrows) in cells of the V2 domain, while coexpression of *Hes6* and *Scl* was not detected (Fig.7 D'-D'''). This last

finding suggests that *Hes6* is not a *Dll4* target, as it is not expressed in prospective V2b INs (*Scl*-expressing cells) where Notch signalling is activated via *Dll4.* Another possibility is that *Hes6* mRNA is no longer expressed when *Scl* expression starts. Concerning *Dll1*, it is known that cells activated through *Dll1*-mediated Notch signalling (*Hes5*$^{+}$*LFng*$^{+}$) can also express *Hes6* [17], therefore it is possible that *Hes6* is a target of *Dll1*-mediated Notch signalling.

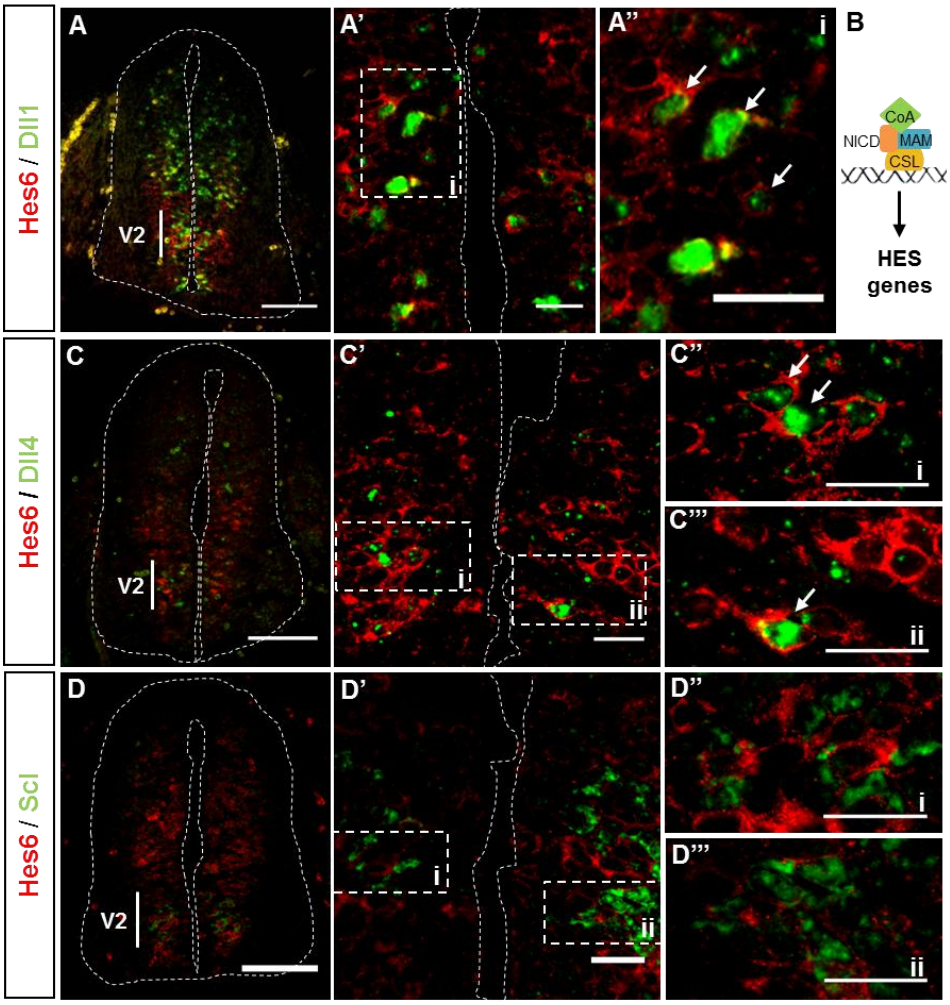

Figure 7 – *Hes6* expression in the V2 domain. (A, C, D) – *Hes6* is expressed in the VZ of V2 domain, in progenitor and differentiating cells. (B) – Schematic representation of activation of HES genes transcription. (A') – *Hes6* is coexpressed with *Dll1* and *Dll4* (C') as the magnifications of the selected regions indicate (A'', C'', C''' – indicated by arrows). (D') – *Hes6* is not coexpressed with *Scl*, a marker for prospective V2b cells. (D'', D''') – magnification of selected regions from confocal image D'.
Scale bars: A, C, D - 50µm; A', A'', C'-C''', D'-D''' - 10µm.

III.2. CHARACTERIZATION OF *Dll4*-EXPRESSING CELLS

Previous results from our lab showed that several regions in the *Dll4* promoter, conserved between mouse and chicken, contain regulatory information for *Dll4* expression. In these regions, several E-boxes are present and are putative binding sites for proneural proteins like MASH1 and Neurogenins (NGNs), which are therefore good candidates to regulate the *Dll4* expression in the V2 domain. Also, it has been reported that *Mash1, Ngn1* and *Ngn2* are expressed in the V2 domain, although the relationship between their expression and that of *Dll4* is still unclear.

In order to see if *Dll4*-expressing cells also express *Mash1*, *Ngn1* or *Ngn2*, double *in-situ* hybridization was performed in neural tubes of E11.5 mice embryos.

As previously published, *Mash1* is expressed in all dorsal spinal cord domains, except in dl6. Ventrally, it is only expressed in V2 domain (Fig.8 A). Both *Ngn1 and Ngn2* are expressed in all ventral domains, being absent in the dorsal most domains (Fig.8 C, E).

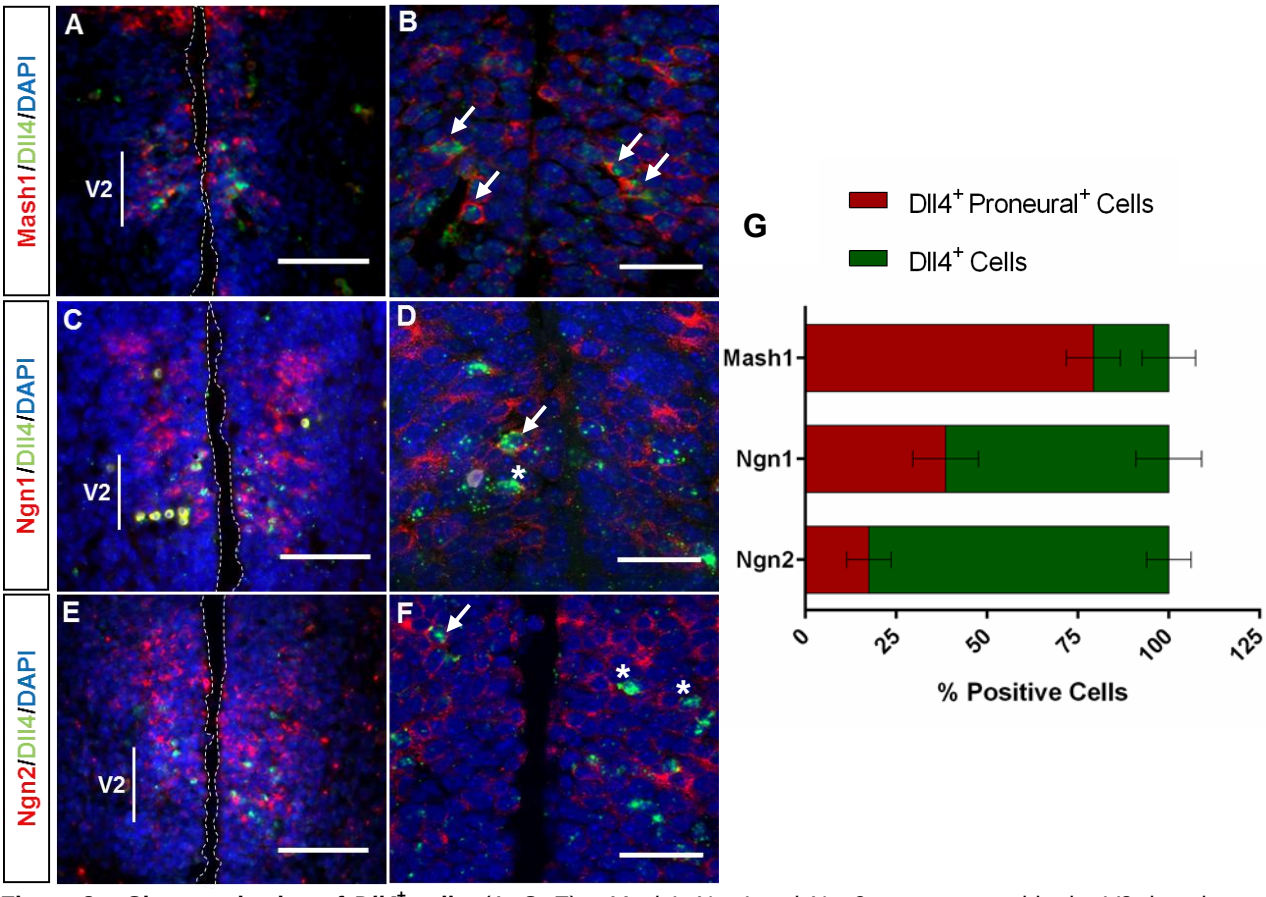

Figure 8 – Characterization of *Dll4*$^+$ cells. (A, C, E) – *Mash1*, *Ngn1* and *Ngn2* are expressed in the V2 domain. (B) – *Mash1* is expressed in the majority of *Dll4*$^+$ cells (white arrows). (D) – *Ngn1* is also expressed in *Dll4*$^+$ cells (white arrow) but some of these cells don't express *Ngn1* (asterisk). (F) – *Ngn2* is the proneural less co-expressed with *Dll4* as many *Dll4*$^+$ cells don't express *Ngn2* (asterisk). (G) – Graphic showing the percentage of *Proneural*$^+$ *Dll4*$^+$ cells as a fraction of the *Dll4* population. 79% of *Dll4*$^+$ cells are also *Mash1*, 39% are also *Ngn1* and 17% are also *Ngn2*. Average from three different sections of five different embryos. Scale bars: A, C and E - 20µm (Fluorescence Microscope); B, D and F - 10µm (Confocal Microscope).

In the V2 domain, the population of *Mash1*$^{+}$ *Dll4*$^{+}$ cells (Fig.8 B – white arrows) corresponds to 79% of the total *Dll4* population (Fig.8 G). Regarding *Ngn1*, less than half of *Dll4*$^{+}$ cells co-express *Ngn1* – 39% (Fig.8 D – arrow, G). Cells that express *Dll4* but do not express *Ngn1* could also be identified (Fig.8 D – asterisk). When analysing results for *Ngn2*, the results show that the majority (83%) of the *Dll4*$^{+}$ cells do not express *Ngn2* (Fig. 8 F – asterisks) with only 17% being *Ngn2*$^{+}$ (Fig.8 F – white arrow, G).

Overall, these results indicate that the three proneural genes are expressed in *Dll4*-expressing cells but to a different extent, with *Mash1* being the most co-expressed gene, followed by *Ngn1* and finally by *Ngn2*. The fact that *Mash1* is the proneural gene that shows higher co-expression with *Dll4* raises the hypothesis that *Mash1* is the major regulator of *Dll4* expression, when compared to both *Ngn1* and *Ngn2*.

III.3. MASH1 OR NGN1 OVEREXPRESSION REPRESSES *Dll4* EXPRESSION IN V2 DOMAIN

To test possible role of *Mash1*, *Ngn1* and *Ngn2* in regulating *Dll4* expression, these transcription factors were overexpressed, single or in combination, in the chick embryonic spinal cord, and their effect in eliciting *Dll4* expression was evaluated. In these experiments, a plasmid encoding the CHERRY fluorescent protein was co-electroporated with the vectors encoding proneural genes, in order to allow the identification of electroporated cells in the spinal cord (see Materials and Methods). Embryos were harvested 36 hours after electroporation and *Dll4* expression analysed, using fluorescent *in-situ* hybridization

Two different positive controls for Dll4 probe specificity were made. It is well known that endothelial cells express high levels of *Dll4* (Fig. 16, Annex B – white arrows), and our results show also strong *Dll4* expression in these cells. In embryos electroporated with Hes6-2:VP16 (dominant-negative form of Hes6-2 protein that act as an activator of *Dll4* expression) we observed *Dll4* expression in the electroporated cells, accordingly to previous reports (Fig. 16, Annex B).

Our results show that MASH1 overexpression represses *Dll4* expression in the V2 domain (Fig. 9 – A', A'', G). This repression is significant and consistent across different embryos (n=8, p=2.512702e-006).

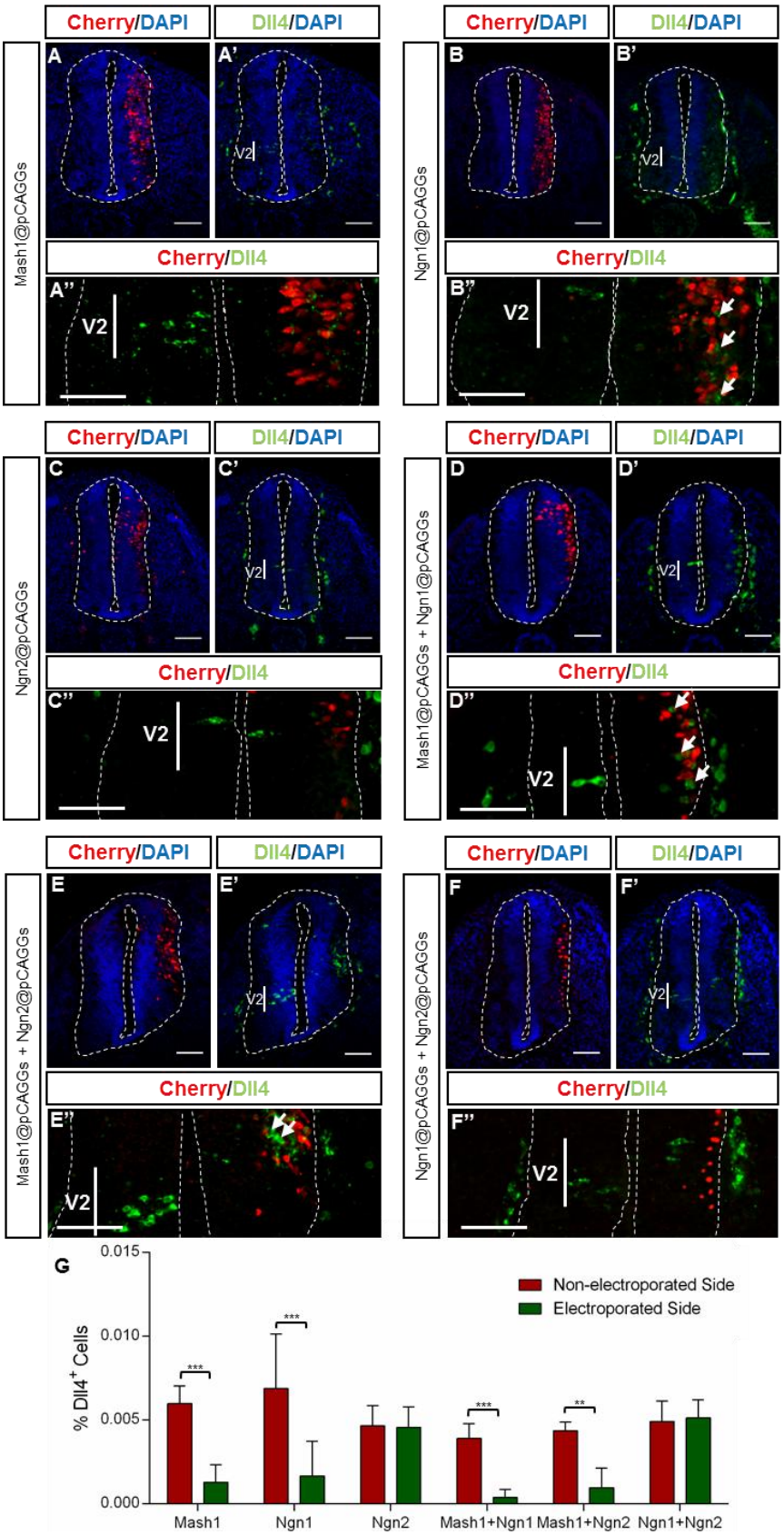

Figure 9 – *Dll4* expression after electroporation of *Mash1*, *Ngn1* and *Ngn2*, single or in combinations. (A-A'') – MASH1 represses *Dll4* expression in the V2 domain. (B-B'') – NGN1 overexpression also represses *Dll4* in the V2 domain but it induces ectopic *Dll4* expression along DV axis (White arrows). (C-C'') – NGN2 overexpression does not change *Dll4* expression. (D-D'') – MASH1 together with NGN1 repress *Dll4* within V2 domain while induce ectopic *Dll4* expression along the DV axis of the spinal cord. (E-E'') – MASH1 and NGN2 also repress *Dll4* expression in the V2 domain and induce it, in the electroporated cell region. (F-F'') – Both NGNs overexpressed do not alter *Dll4* expression in the developing spinal cord. (G) – Percentage (%) of *Dll4*$^{+}$ cells between sides of the spinal cord in each one of the conditions tested (proneurals overexpression). Statistical significance (*** - *p-value* < 0,01; ** - *p-value* < 0,05). In all embryos, Cherry protein was used to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency (A, B, C, D, E, and F). Embryos were harvested 36 hours after electroporation. Scale bars: A-F'' - 50µm

Overexpression of NGN1 also represses *Dll4* expression in the V2 domain (Fig.9 – B', B'', G; *n*=8, *p*=0.00383657) however it induces ectopic *Dll4* expression in other domains along the DV axis of the spinal cord (Fig. 9 – B'', white arrows). The observed induction of *Dll4* expression occurs mostly in a non-cell autonomous manner (cells next to the electroporated ones).

Overexpression of NGN2 does not alter *Dll4* expression in the V2 domain, neither causes ectopic expression in other domains (Fig. 9 – C-C''', G; *n*=8, *p*=0.89066).

These results show that MASH1 and NGN1 are able to regulate *Dll4* expression, while NGN2 is not.

To test for the presence of combinatorial effects in regulation of *Dll4* expression, MASH1, NGN1 and NGN2 were overexpressed in different combinations.

Simultaneous overexpression of MASH1 and NGN1 causes a repression of *Dll4* expression in the V2 domain (Fig. 9 – D-D''', G; *n*=3, *p*=0.00356786), while inducing ectopic expression of *Dll4*, dorsally to V2, (Fig. 9 D''' – white arrows), in a non-cell autonomous manner.

Simultaneous overexpression of MASH1 and NGN2 also represses *Dll4* expression (Fig. 9 – E-E''', G; n=3, *p*=0.0103982) while induces ectopic expression (Fig. 9 – E''', white arrows).

When both NGNs are overexpressed, there are no significant differences in the number of *Dll4*-expressing cells (Fig. 8 – F-F''', G), comparing both sides of the spinal cord (*n*=3, *p*=0.828263).

In conclusion, our data, from simultaneous overexpression of proneural genes, suggest that there are no additive effects in regulation of *Dll4* expression by proneural proteins. However NGN2 can overcome NGN1 function, as simultaneous overexpression of both NGNs mimics the effect of single overexpression of NGN2 - *Dll4* expression is not altered.

III.3. MASH1 OVEREXPRESSION LEADS TO A DECREASE IN V2A INS WHEREAS NGN1 AND NGN2 OVEREXPRESSION HAS THE OPPOSITE EFFECT

Parras et. al and Li et. al reported that proneural proteins can affect IN specification inside V2 domain. To determine whether overexpression of proneural proteins affects V2a interneuron specification, embryos that were electroporated with plasmids encoding these proteins, were analysed for the expression of CHX10, a protein specifically expressed in V2a INs, using immunofluorescence with an appropriate antibody.

After MASH1 overexpression, a decrease in the number of CHX10$^+$ cells is observed in the electroporated side, when compared with the non-electroporated side of the spinal cord (Fig. 10 A-A', G; *n*=8, *p*=6.364437e-011).

After NGN1 or NGN2 overexpression, an increase in the number of V2a INs (CHX10$^+$ cells) is observed in the electroporated side of the spinal cord (Fig. 10 B-B' and C-C', respectively). The increase of CHX10$^+$ cells in the electroporated side were significant for NGN1 (*n*=8, *p*=7.425886e-011) and for NGN2 (*n*=8, *p*=5.198783e-013).

Overall, these observations indicate that MASH1 represses V2a IN fate whereas NGN1 and NGN2 promote this fate.

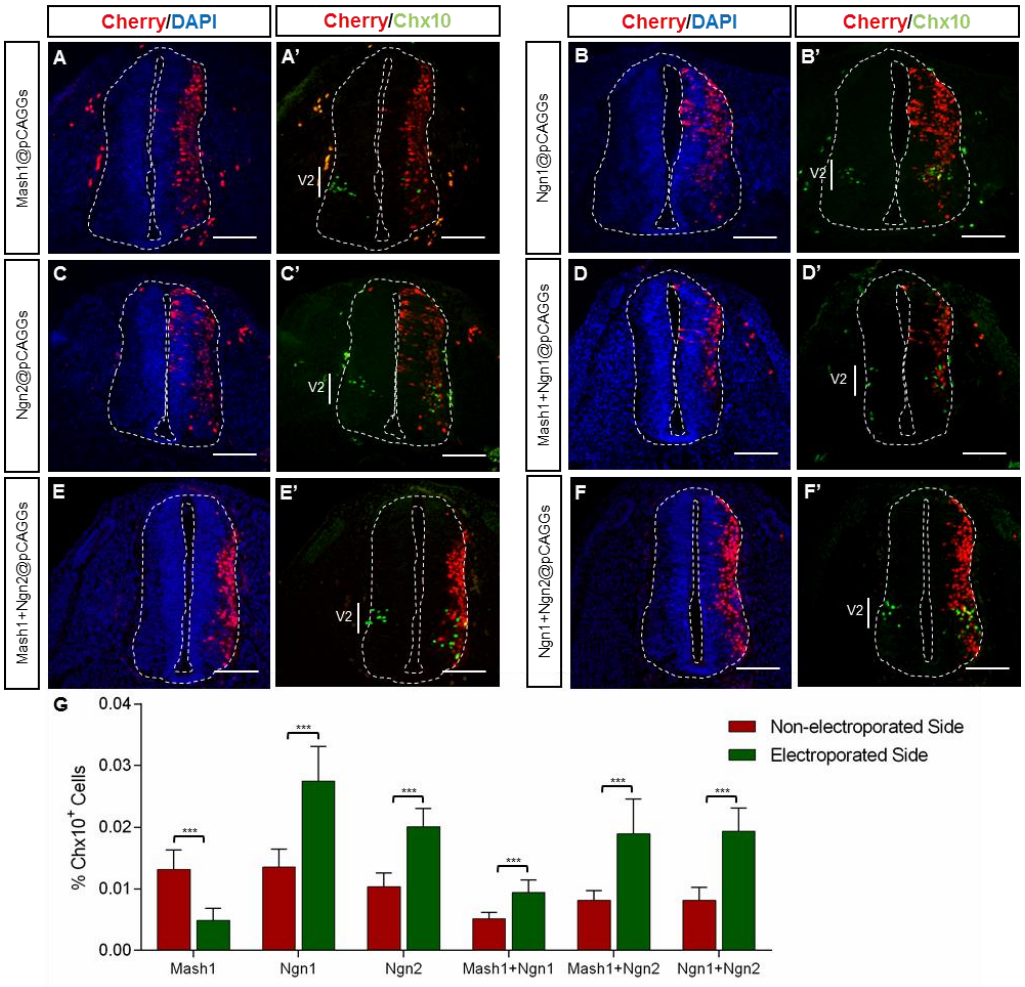

Figure 10 – CHX10 expression after electroporation of *Mash1*, *Ngn1* and *Ngn2*, single or in combinations. (A-A'') – MASH1 overexpression leads to a decrease in the number of CHX10$^+$ cells in the electroporated side of the spinal cord. (B-B'') – NGN1 overexpression leads to an increase in CHX10$^+$ cells in the electroporated side of the spinal cord. (C-C'') – NGN2 overexpression also leads to an increase in the number of CHX10$^+$ cells. (D-D'') – MASH1 together with NGN1 increases CHX10$^+$ cells in the electroporated side of the spinal cord. (E-E'') – MASH1 and NGN2 also increases the number of CHX10$^+$ cells within the electroporated side. (F-F'') – Both NGNs overexpressed increase CHX10$^+$ cells in the electroporated side of the spinal cord. (B', C', E' and F') – Most of the CHX10-positive cells in the electroporated side correspond to electroporated cells (cherry-positive cells). (G) – Percentage (%) of CHX10$^+$ cells between sides of the spinal cord in each one of the conditions tested (proneurals overexpression). Statistical significance (*** - *p-value* < 0,01). In all embryos, Cherry protein was used to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency (A, B, C, D, E, and F). Embryos were harvested 36 hours after electroporation. Scale bars: A-F'' - 50µm.

In order to dissect for the presence of combinatorial effects in regulation of IN specification, MASH1, NGN1 and NGN2 were overexpressed in different combinations.

When MASH1 was overexpressed with NGN1, an increase in the number of $CHX10^+$ cells is observed in the electroporated side of the spinal cord (Fig. 10 D-D', G; n=3, p=0.00347376).

Simultaneous overexpression of MASH1 and NGN2 or NGN1 and NGN2 increases the number of $CHX10^+$ cells (Fig. 10 E-E and F-F'', G). Increase of V2a INs in the electroporated side of the spinal cord were significant for MASH1 and NGN1 (n=3, p=0.00384054) and for NGN1 and NGN2 (n=3, p=0.000398324).

These observations indicate that positive regulation by NGNs can overcome the repressive effects of MASH1, as simultaneous overexpression of MASH1 with NGN1 or NGN2 can increase the number of V2a INs. When both NGNs were electroporated the increase in V2a INs was not higher than in the single electroporation scenario so we can exclude an additive effect in regulation of V2a IN fate.

III.4. MASH1 AND NGN1 OVEREXPRESSION LEADS TO A DECREASE IN THE NUMBER OF PROSPECTIVE V2a

The combined expression of *Lim3* and *bhlhb5* was next used to identify an earlier stage of V2a development, before these cells express the differentiation marker *Chx10*. While *Lim3* is expressed in all V2 progenitors, *bhlhb5* is specifically expressed in V2a INs, allowing us to ask whether the increase in CHX10 V2a Ins is due to an increase in the number of V2 progenitors ($Lim3^+$) that commit to V2a fate ($Lim3^+bhlhb5^+$) or, alternatively, to an acceleration in the differentiation of these progenitors.

To test this, combined expression of bHLHB5 and LIM3 was analysed in embryos electroporated with proneural genes, using immunofluorescence with the appropriate antibodies.

The results show that overexpression of MASH1 results in a decrease of $bHLHB5^+LIM3^+$ cells (Fig.11 A-A'', G; n=4, p= 4.339579e-010). Also in this condition, less $bHLHB5^+LIM3^+$ cells were detected inside the ventricular zone (Fig.11 A'' – white arrow). No differences were detected regarding the total number of $Bhlhb5^+$ cells in all expressing domains (dl6, V1and V2).

After NGN1 overexpression, a decrease in the number of bHLHB5^{+}LIM3^{+} cells is observed (Fig.11 B-B'',G; *n*=4, *p*= 2.847715e-005). Like in MASH1 overexpression condition, just a small number of BHLHB5^{+}LIM3^{+} cells were detected inside the VZ (Fig.11 B'' – white arrow). An increase in the number of bHLHB5^{+} cells were detected in the electroporated side of the spinal cord, across all domains where this gene is normally expressed.

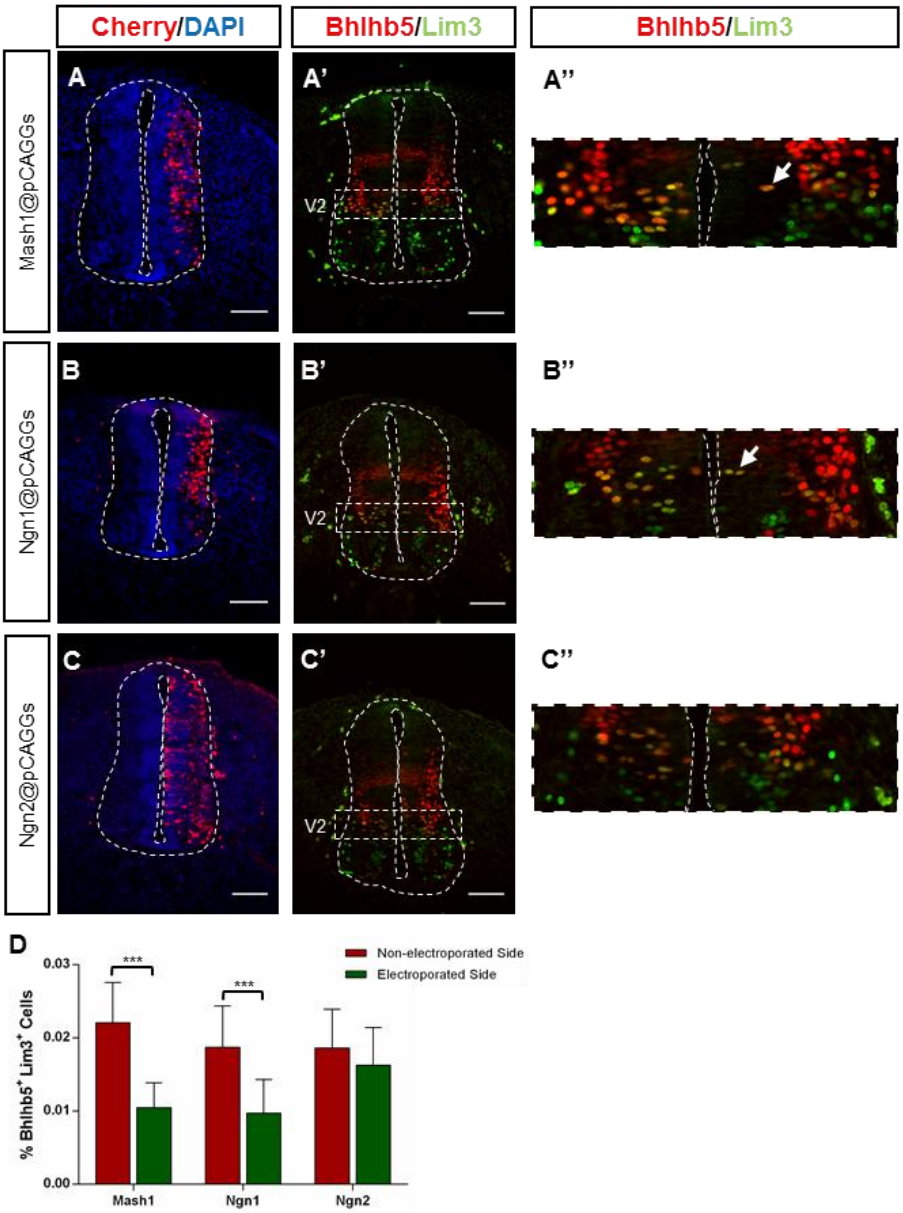

Figure 11 – bHLHB5 and LIM3 expression after electroporation of *Mash1*, *Ngn1* and *Ngn2*. (A-A'') – MASH1 overexpression leads to a decrease in the number of bHLHB5^{+}LIM3^{+} cells, inside the V2 domain. (B-B'') – NGN1 overexpression decreases the number of these double^{+} cells. (C-C'') – NGN2 does not alter the number of bHLHB5^{+}LIM3^{+} cells in the electroporated side of the spinal cord. (D) - % of bHLHB5^{+}LIM3^{+} cells between sides of the spinal cord in each one of the conditions tested (proneurals overexpression). Statistical significance (*** - *p-value* < 0,01). In all embryos, Cherry protein was used in adjacent sections to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency (A, B and C). Embryos were harvested 36 hours after electroporation. Scale bars: A-F - 50µm.

NGN2 overexpression does not affect the number of bHLHB5$^+$LIM3$^+$ cells (Fig.11 C-C'', G), as no differences were detected between sides of the spinal cord (*n*=4, *p*=0.259507). Furthermore no differences in the bHLHB5$^+$ population were detected.

These observations indicate that overexpression of MASH1 or NGN1 can affect the number of prospective V2a inside the V2 domain. MASH1 overexpression leads to a decrease in bHLHB5$^+$LIM3$^+$ progenitors and differentiating CHX10$^+$ V2a. NGN1 causes also a decrease in bHLHB5$^+$LIM3$^+$ progenitors but an increase in CHX10$^+$ V2a INs. These results suggest that while MASH1 "freezes" progenitors in an undifferentiated state, NGN1 accelerates neuronal differentiation and causes a depletion of progenitors. In contrast, NGN2 not affects the prospective V2a IN population.

III.5. MASH1, NGN1 AND NGN2 OVEREXPRESSION LEADS TO A DECREASE IN V2B IN FATE

To determine how overexpression of proneural proteins affects V2b interneuron specification, embryos were electroporated with plasmids encoding these proneural proteins and harvested 36 hours after electroporation. To identify prospective V2b INs, the expression of *Scl* was analysed, using RNA *in-situ* hybridization.

The results show that MASH1 overexpression causes a decrease in the number of *Scl*$^+$ V2b cells (Fig. 12 A-A', G). In all embryos analysed, the decrease was striking with almost no *Scl*$^+$ cells in the electroporated side (*n*=8, *p*=8.314637e-018).

NGN1 or NGN2 overexpression, also causes a decrease in the number of *Scl*$^+$ V2b cells (Fig. 12 B-B' and C-C', G). This decrease was significant for NGN1 (*n*=8, *p*=1.449310e-013) and for NGN2 (*n*=8, *p*=8.226599e-008).

These data point out that all three proneural proteins tested repress the prospective V2b IN fate, with MASH1 repression being the strongest one, as almost no *Scl*$^+$ cells could be detected in the electroporated side of the spinal cord.

In order to dissect for the presence of combinatorial effects in regulation of IN specification, MASH1, NGN1 and NGN2 were overexpressed in different combinations.

When MASH1 was overexpressed with NGN1 a decrease in the number of *Scl*$^+$ cells is observed (Fig. 12 D-D', G). Simultaneous overexpression of MASH1 and NGN2 or NGN1 and NGN2 overexpression, causes a decrease in the number of *Scl*$^+$ V2b cells (Fig. 12 E-E' and F-F', G). The observed decrease in the *Scl*$^+$ V2b cell population in the electroporated side of the spinal cord was significant for MASH1 and NGN1 (*n*=3,

p=6.615791e-009), for MASH1 and NGN2 (*n*=3, *p*=9.457499e-007) and for both NGNs (*n*=3, *p*=9.495541e-007).

Overall these results indicate that the three tested proneural proteins repress the *Scl*$^{+}$ V2b cells. We could not detect an additive effect or proneural proteins function as, in none of the simultaneous overexpression conditions we detect a higher repression effect than in single overexpression conditions. However the marker used in these analyses (*Scl*) is transiently expressed during V2 cell transitions, so the number of V2b cells could be underestimated, as *Scl* is not a final differentiation marker, as CHX10 (V2a analysis).

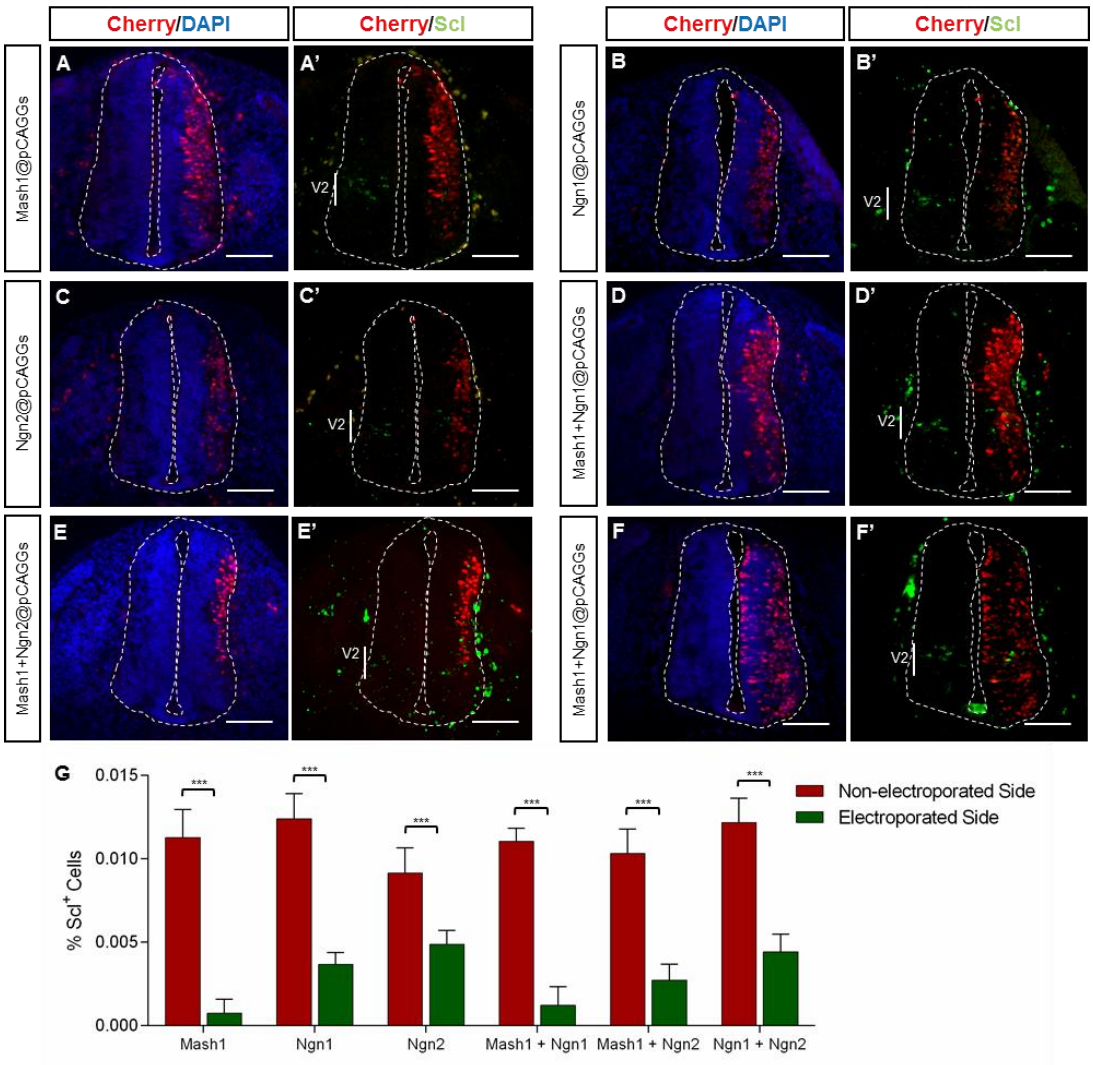

Figure 112 – *Scl* expression after electroporation of *Mash1*, *Ngn1* and *Ngn2*, single or in combinations. (A-A'') – MASH1 overexpression leads to a decrease in the number of *Scl*$^{+}$ cells in the V2 domain. (B-B'') – NGN1 overexpression also represses the number of *Scl*$^{+}$ cells. (C-C'') – NGN2 overexpression also decreases the number of *Scl*$^{+}$ cells. (D-D'') – MASH1 together with NGN1 represses *Scl*$^{+}$ cells within V2 domain. (E-E'') – MASH1 and NGN2 overexpression decreases the number of *Scl*$^{+}$ cells in the electroporated side of the spinal cord. (F-F'') – Both NGNS overexpressed decrease *Scl*$^{+}$ cells in the developing spinal cord. (G) – Percentage (%) of *Scl*$^{+}$ cells between sides of the spinal cord in each one of the conditions tested (proneurals overexpression). Statistical significance (*** - *p-value* < 0,01). In all embryos, Cherry protein was used to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency (A, B, C, D, E, and F). Embryos were harvested 36 hours after electroporation. Scale bars: A-F'' - 50µm.

III.6. SINGLE MASH1 OVEREXPRESSION AFFECTS DV SPECIFICATION OF THE SPINAL CORD

Proneural genes are affecting *Dll4* expression and IN specification inside the V2 domain. It is known that the correct positioning of spinal cord domains is achieved through different morphogen combinations that will establish the DV axis of the spinal cord. Neuronal differentiation, in the spinal cord, involves crossregulatory interactions between a bHLH-driven program of neurogenesis and genetic pathways of DV patterning that specify progenitor and neuronal identity. For instance, it was shown that Retinoic acid, via Notch signalling, control the expression of *Mash1* and that quantitative differences of this transcription factor can select distinct fates [52].

Our hypothesis is that overexpression of proneural genes can impose a new subtype specification program on progenitors and, therefore, affect the DV specification of the spinal cord. We have focused in the MN domain because it was shown that the MN domain maintains cross-antagonistic interactions with V2 domain [29].

To test this, we analysed the expression of OLIG2, a protein specifically expressed in MN progenitors. It was also analysed JAG1 expression, a Notch ligand expressed in V1 domain, in order to have an internal control for DV modifications between both sides of the spinal cord. Embryos electroporated with plasmids encoding these proneural proteins, were analysed for the combined expression of OLIG2 and JAG1, using immunofluorescence with appropriate antibodies.

When MASH1 was overexpressed, an increase in the number of $OLIG2^+$ cells is observed (Fig. 13 – A and G), being this increase significant (n=4, p= 0.000812698).

After NGN1 overexpression, no differences in the $OLIG2^+$ population were observed (n=4, p= 0.0760502; Fig. 13 – B and G). NGN2 overexpression does not alter the number of $OLIG2^+$ cells (n=3, p= 0.0760502; Fig. 13 – C and G).

To dissect for the presence of combinatorial effects in regulating DV specification, MASH1, NGN1 and NGN2 were overexpressed in different combinations.

Simultaneous MASH1 and NGN1 overexpression leads to an increase in $OLIG2^+$ cells (n=3, p=0.00413902; Fig. 13 – D and G). When MASH1 was overexpressed with NGN2 an increase in the $OLIG2^+$ population was noticed (n=3, p= 0.0407129; Fig. 13 – E and G). When both NGNs were overexpressed, the same trend was observed (n=3, p= 0.0165837; Fig. 13 – F and G).

These observations indicate that MASH1 is the most effective proneural in regulating the number of $OLIG2^+$ cells (MN progenitors). The excess of $OLIG2^+$ cells could indicate that these cells are being produced at the expense of V2 progenitors or at the expense of fully differentiated V2 INs. MASH1 could be imposing a new subtype

specification program on V2 progenitors and changing their fate towards a MN fate, affecting the DV specification of the spinal cord. Simultaneous overexpression of proneural genes has the same effect, increasing the number of MN progenitors (pMN).

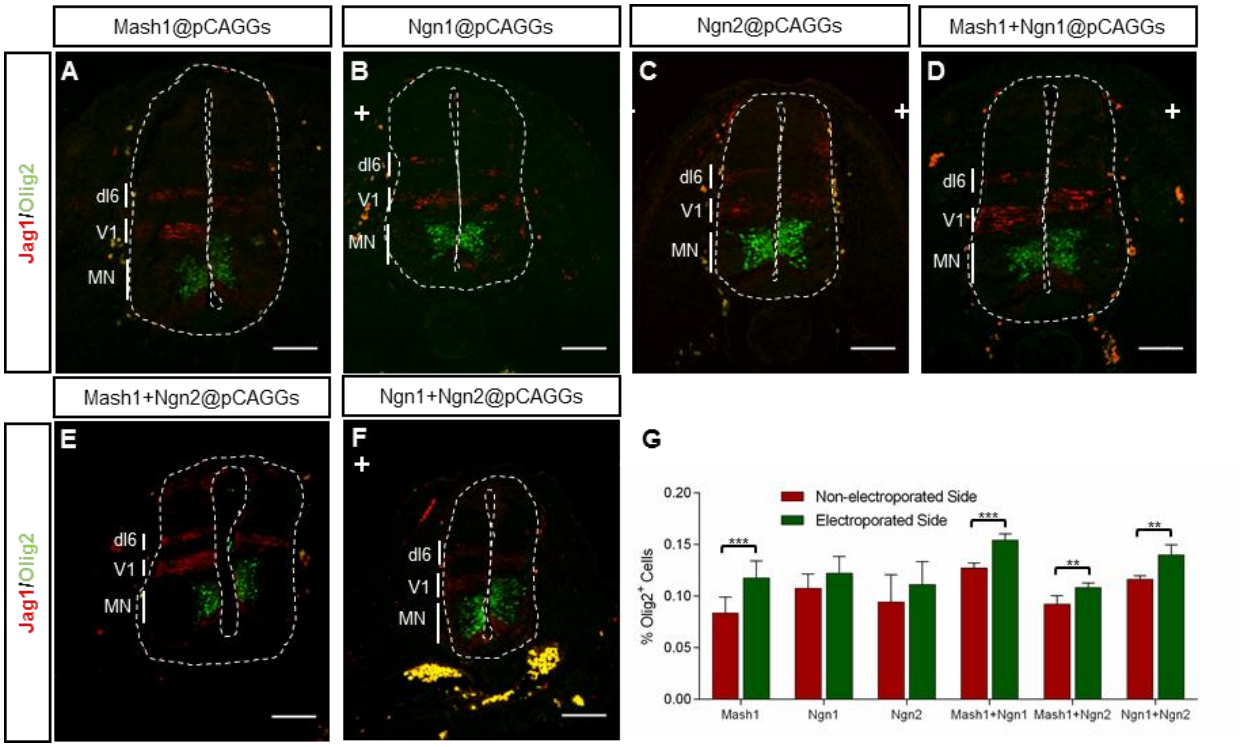

Figure 123 - JAG1 and OLIG2 expression after electroporation of *Mash1*, *Ngn1* and *Ngn2*, single or in combinations. (A) – MASH1 overexpression leads to an increase in the OLIG2$^+$ cells. (B, C) – NGN1 or NGN2 overexpression does not alter the number of OLIG2$^+$ cells. (D) – MASH1 and NGN1 overexpression increase the number of OLIG2$^+$ cells. (E) – MASH1 and NGN2 overexpression also increase OLIG2$^+$ cells. (F) – NGN1 and NGN2 overexpression has the same effect in the OLIG2$^+$ population. (G) – Percentage (%) of OLIG2$^+$ cells between sides of the spinal cord in each one of the conditions tested (proneurals overexpression). Statistical significance (*** - *p-value* < 0,01; ** - *p-value* < 0,05*)*. In all embryos, Cherry protein was used in adjacent sections to identify the electroporated side of the spinal cord and as a positive control of the electroporation efficiency. Embryos were harvested 36 hours after electroporation. (+) – Identification of the electroporated side of the spinal cord. Scale bars: A-F - 50µm.

To test whether apoptosis is occurring after electroporation and affects the results above described, an antibody against activated caspase 3 was used to detect apoptotic cells in adjacent sections of electroporated spinal cords. No significant differences were found in the number of apoptotic cells between the electroporated and non-electroporated sides of the spinal cord (data not shown), revealing that the observed alterations are not due to non-specific apoptotic effects.

In all analysis, single overexpression of proneural proteins results from an average of four sections on six embryos while simultaneous overexpression of proneural proteins results from an average of four sections on two or three embryos.

CHAPTER IV

Discussion

CHAPTER IV – DISCUSSION

The CNS is a complex system composed by different types of cells however the mechanisms responsible for the generation of this cell diversity are not fully understood. It is known that Notch signalling is important in this process and this project presents new data on how the expression of one Notch ligand, Dll4, is regulated and might be involved in IN specification in the V2 domain of the chick developing spinal cord.

IV.1. NOTCH SIGNALLING IN THE V2 DOMAIN

In the developing spinal cord, the V2 domain is the only one where two Notch ligands are expressed (DLL1 and DLL4). To unravel if *Dll1*-mediated Notch signalling is different than *Dll4*-mediated Notch signalling, we analysed the expression of two different genes (*LFng* and *Hes6*). *LFng* is a potentiator of Notch signalling, while *Hes6* is a Notch signalling target that releases proneural genes from the inhibitory action of *Hes5*. These genes enable us to see if, in fact, *Dll1* and *Dll4*-mediated Notch signalling could be different, in the V2 domain.

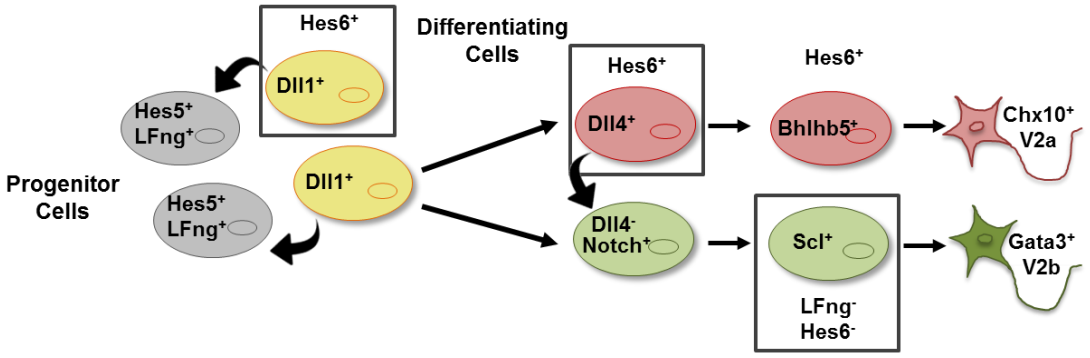

Figure 13 – Simplified model of cell transitions in the V2 domain. *Dll1*-expressing cells express *Hes6*. These cells are starting the differentiation process, keeping the surrounding cells as progenitors (*Hes5*$^+$ and *LFng*$^+$). The *Dll1*$^+$ cell can upregulate the expression of *Dll4,* will express *Bhlhb5* and then become a V2a IN (*Chx10*$^+$), while the *Dll4*$^-$ ones will express *Scl* and become V2b IN (*Gata3*$^+$). Grey squares represent particular steps of these cellular transitions filled by my data.

It is known that a cell starting the differentiation process will have high levels of *Dll1*, thereby activating Notch in neighbours to keep these as progenitors (*Hes5*$^+$*LFng*$^+$). We show that *LFng* can be expressed in *Dll4*-expressing cells in the IVZ. We demonstrate that cells that receive *Dll4* signalling (and become *Scl*$^+$ V2b) are not expressing *LFng*, so Notch is activated by *Dll4* in *Scl*$^+$ cells without *LFng* potentiator (Figure 14). Thus, *Dll4*-mediated Notch signalling is independent of *LFng*. As described previously, *Dll1* is keeping the surrounding cells as progenitors, which express *LFng*, so *Dll1*-mediated Notch signalling is *LFng* dependent. In the V2 domain, we show that

Dll1 and *Dll4*-mediated Notch signalling are different. We assume that this difference could be due to *LFng* dependence.

The analysis of *Hes6* expression, a downstream target of Notch signalling, shows that it is expressed in *Dll1* and *Dll4*-expressing cells. Since *Dll* ligands are positively regulated by proneural genes, cells that express *Dll1* and/or *Dll4* should also express *Hes6* in order to release these proneural genes from the inhibitory action of other targets of Notch signalling (*Hes5*) and therefore differentiate (Figure 14).

Hes6 may not be a downstream target of *Dll4*, as it is not expressed in prospective V2b INs (*Scl*-expressing cells), where Notch signalling is activated by *Dll4*. Alternatively *Hes6* mRNA could be transient and no longer expressed when *Scl* expression starts. Concerning *Dll1*, it is known that progenitors activated through *Dll1*-mediated Notch signalling can also express *Hes6* [17], therefore it is possible that *Hes6* is a target of *Dll1*-mediated Notch signalling. In the V2 domain, *Dll1* and *Dll4*-mediated Notch signalling are different. The difference could also rely on *Hes6* expression.

In summary, my results indicate that *Dll1* and *Dll4*-mediated Notch signalling inside the V2 domain occur through different molecular components, as *LFng* and *Hes6* are differently expressed in cells where Notch signalling is activate, via *Dll1* or *Dll4*. However, further analysis must be made in order to better characterize *Dll4*-mediated Notch signalling. Notch signalling activated via *Dll4* could be potentiated by other members of the *Fng* family, like *MFng* (*Maniac fringe*) or *RFng* (*Radical fringe*). Also, the difference between *Dll1* and *Dll4*-mediated Notch signalling could rely on other downstream targets of this pathway, like other members of the HES family.

IV.2. Dll4 REGULATION

Several regions in the *Dll4* promoter, conserved between mouse and chicken, contain regulatory information for *Dll4* expression, as previous results from our lab demonstrated [32]. The most proximal conserved region contains a Mash1/Brn motif, previously identified by Castro et. al [26]. This motif is composed by an E-box (CAGCTG) and an evolutionary conserved octamer that is the binding site for the POU family of homeodomain proteins, such as Brn proteins. Another E-box (CAGGTG) was detected and corresponds to the consensus binding sequence for MASH1 [32]. Furthermore, three conserved E-boxes (CAGATG), believed to be preferred for the binding of Neurogenins (NGNs), were found [32]. These results suggest that MASH1, NGN1 and NGN2 could regulate *Dll4* expression in the V2 domain of the chick developing spinal cord.

In order to test this, we performed a gain-of-function approach where MASH1, NGN1 and NGN2 were overexpressed in the chick developing spinal cord, to measure their ability to regulate *Dll4* expression.

Table 1 – Results summarized regarding *Dll4* expression and IN specification in different conditions (single or simultaneous overexpression of proneural proteins).

Condition	Mash1	NGN1	NGN2	Mash1+NGN1	Mash1+NGN2	NGN1+NGN2
Dll4	↓	↓	=	↓	↓	=
Chx10 (V2a)	↓	↑	↑	↑	↑	↑
Scl (V2b)	↓	↓	↓	↓	↓	↓
Ectopic Dll4	**No**	**Yes**	**No**	**Yes**	**Yes**	**No**

The results show that MASH1 is able to repress *Dll4* expression inside the V2 domain (Figure 9 – A', A'' and summarized inTable1). However, no induction of ectopic *Dll4* expression was detected (Table 1), contrarily to previous reports [30, 32, 42]. This discrepancy might be due to the different stages used for electroporation, since previous results [30, 32, 42] analysed MASH1 effects on *Dll4* at earlier stages (HH19). It is possible that the reported ectopic induction of *Dll4* is not observed at HH24-25 (stages analysed during this project).

Concerning the effect of NGN1, our results suggest that it is able to repress *Dll4* expression inside the V2 domain, while it induces ectopic expression in the spinal cord (along the DV axis) (Figure 15; Table 1). In contrast, NGN2 overexpression has no effect on *Dll4* expression, suggesting that this transcription factor is not regulating *Dll4* expression (Figure 15; Table 1). Even though NGN1 and NGN2 are likely to bind to the same consensus E-box sequences (CANATG) [9], only NGN1 is able to activate *Dll4* expression.

This difference between the two NGNs could be due to specific interactions with different co-factors that might affect their transcriptional activity on the *Dll4* promoter. For instance, in cortical development, LMO4 and its binding partner nuclear LIM interactor (NLI/LDB1/CLIM2) form a multi-protein complex with NGN2 that is recruited to the E-box that contains enhancers of NGN2-target genes, activating NGN2-mediated transcription [53]. The reported existence of a strong post-translational control of NGN2 might also explain why overexpression of this transcription factor has no effect on *Dll4* expression: it has been shown, for example, that phosphorylation of NGN2 differentially controls the activation of the *NeuroD* and *Delta* promoters by exploiting differences in the kinetics of activation in response to promoter occupancy by NGN2 [54 55]. Phosphorylated NGN2 would predominate in rapidly cycling cells, resulting in NGN2 - E

protein dimers with weak promoter affinity. This is insufficient to activate *NeuroD* promoter, however is still able to activate *Delta* promoter, resulting in non-cell autonomous Notch-mediated progenitor maintenance [54].

In summary, we show that *Dll4* is regulated by MASH1 and NGN1 but not by NGN2.

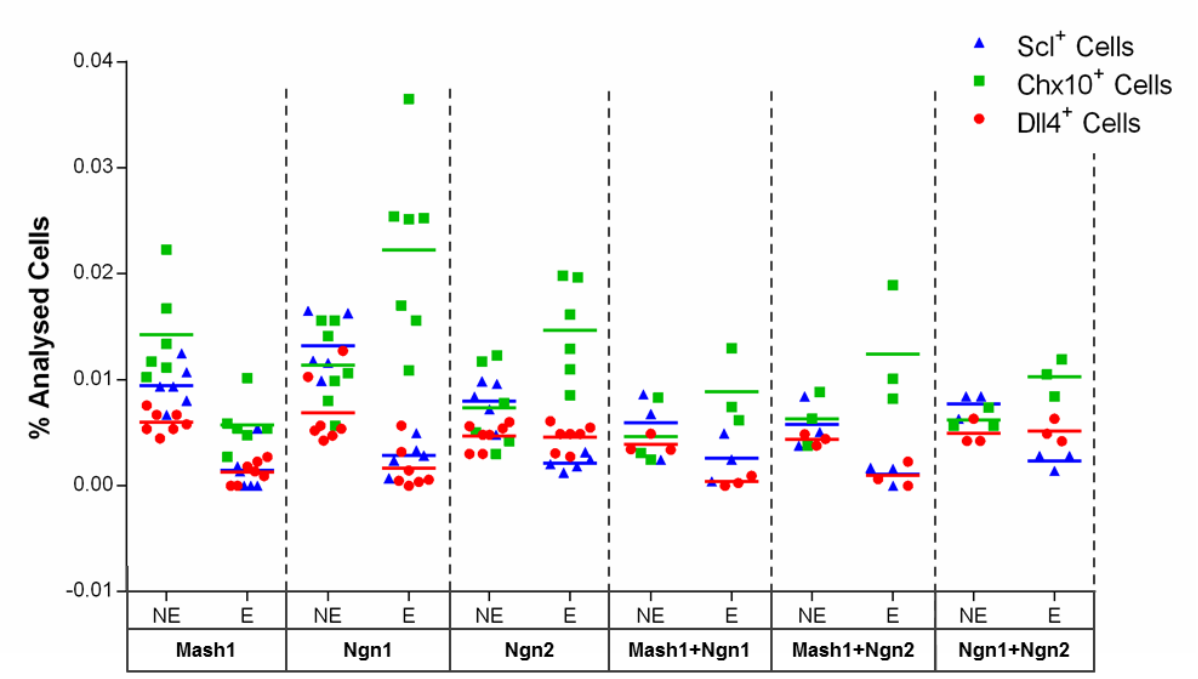

Figure 14 – Summary data regarding % of analysed cells for each condition of proneurals overexpressed. Each embryo analysed is represented by a symbol: red dots, green squares or blue triangles. NE – non-electroporated side of the spinal cord. E – electroporated side of the spinal cord.

If we simply consider the expression pattern of these proneural genes in the V2 domain, we can distinguish earlier and later phases of expression with a sequential action. *Mash1* and *Ngn1* are expressed in cells closer to the lumen of the spinal cord in the IVZ and thus can be considered as "earlier" expressed, while *Ngn2* is expressed in cells located more basally and it can be considered as a "later" proneural, acting after *Mash1* and *Ngn1*.

Our data, in mouse embryos, show that *Mash1* and *Ngn1* are the proneural genes that are more co-expressed with *Dll4* (79% and 39%, respectively). Due to their higher co-expression in *Dll4*-expressing cells and as early proneural genes, we propose that *Mash1* and *Ngn1* could have a major role in regulating *Dll4* expression, while *Ngn2*, as a late proneural gene, could have a major role in V2 IN specification.

IV.3. V2 INTERNEURON SPECIFICATION

Besides regulating *Dll4* expression, it has been shown that MASH1, NGN1 and NGN2 can also regulate neuronal specification. For instance Parras et. al [19] and Li et.

al [48] have shown that proneural proteins are important for the specification of V2a and V2b INs. Since proneural proteins can regulate neuronal subtype specification, we analysed if overexpression of MASH1, NGN1 or NGN2 affects the number of V2 INs produced.

Concerning MASH1 function, our data shows that it can produce a decrease in the number of CHX10$^+$ V2a INs and prospective *Scl*$^+$ V2b INs, in agreement with Li et al. [48]. Thus, MASH1 is repressing both V2a and V2b IN fate, cell autonomously, as the few CHX10$^+$ cells or *Scl*$^+$ cells that appear in the electroporated side correspond to non-electroporated cells (Table 1, Figure 15).

Regarding the role of NGN1, it is increasing the number of V2a INs (Table 1, Figure 15). As most of the CHX10$^+$ cells correspond to electroporated cells, a possible explanation is that misexpressed NGN1 can act cell-autonomously to promote this fate, independently of Notch signalling activation in these cells.

NGN2 overexpression, although it does not alter *Dll4* expression, increases the number of V2a INs (Table 1, Figure 15). As most of CHX10$^+$ cells correspond to electroporated cells, these results suggest that NGN2 is acting cell-autonomously to promote the V2a IN fate. Once again, if we consider NGN2 as a late proneural gene, it is affecting IN specification without affecting *Dll4* expression.

The results also suggest that single overexpression of either NGN1 or NGN2 is decreasing the number of prospective V2b INs. The two NGNs might act cell-autonomously to repress this fate, as *Scl*$^+$ cells in the electroporated side correspond to non-electroporated cells (Table 1, Figure 15). NGN1 and NGN2 could be accelerating differentiation of V2b INs, as few prospective V2b are observed. Another possibility is that both NGNs are promoting V2a IN fate at the expense of V2b INs. Further analysis of the fully differentiated V2b INs (Gata3$^+$ cells) should be done, in order to validate this hypothesis.

Concerning simultaneous overexpression of proneural proteins, we see that NGNs are more efficient in promoting V2a fate as they overcome MASH1 effect (Figure 15). However, it should be mentioned that the number of embryos analysed was lower than in single overexpression conditions.

After single overexpression of either NGN1 or NGN2, some CHX10$^+$/CHERRY$^+$ cells were observed, ventrally to the V2 domain, a position where differentiated V2 INs usually migrate to. It is possible that NGN1 and NGN2 are promoting the V2a IN fate, causing these cells to prematurely differentiate. Alternatively, NGN1 and NGN2 might be inducing ectopic V2a INs in the MN and/or V3 domains.

Our hypothesis of early and late proneural genes would imply that MASH1 and NGN1 (early proneurals) regulate *Dll4* expression, while late proneural genes affect

just the IN specification. Our results show, indeed, that NGN2, as a later proneural, only affect IN specification without affecting *Dll4* expression inside the V2 domain.

IV.4. PRONEURAL GENES MISEXPRESSION AND NEUROGENESIS

The results previously presented show that MASH1 is able to repress *Dll4* expression and, also, the number of V2a and V2b INs. A decrease in the number of prospective V2a IN ($BHLHB5^{+}LIM3^{+}$) was also reported, which supports the observed decrease in the number of V2a INs. Concerning Mash1 overexpression, the number of $BHLHB5^{+}$ cells also decreases in the V2 domain, thus suggesting that MASH1 is keeping progenitors in an undifferentiated state.

We hypothesise that MASH1 misexpression might be keeping progenitors proliferative and therefore affects the rate of neurogenesis in this domain. This could explain the low number of $Dll4^{+}$ cells and also V2a and V2b INs, when MASH1 is overexpressed. Ultimately this suggests that neurogenesis is happening at a lower rate comparing with the non-electroporated side of the spinal cord. To confirm this hypothesis, analysis with different neuronal (TUJ1) and progenitor markers (*Sox* genes or *Hes5*) should be done.

Previous reports show that MASH1 activates a large number of positive cell cycle regulators. Loss-of-function analysis *in vivo* confirmed that *Mash1* plays a major role in normal progenitor divisions. So besides regulating early steps of neurogenesis, including neuronal commitment, subtype specification and cell cycle exit of progenitors, *Mash1* can also promote cell proliferation [56]. *Mash1* is indeed required for normal proliferation of neural progenitors in telencephalic development [56] but also in adult mouse brain by maintaining subventricular zone cells with long term neurogenic potential [57]. This function appears to be evolutionarily conserved as *Asense*, *Mash1* ortholog in *Drosophila*, also promotes neuroblasts self-renewal [58]. To confirm this hypothesis, in MASH1 misexpressed embryos, *Sox2* (progenitor analysis), *Ki67* or BrdU injections (proliferation analysis) should be done.

Besides MASH1 function in regulating *Dll4* expression and IN specification, our results show also an increase in the number of OLIG2-positive cells (progenitors of MN domain) in MASH1 misexpressed embryos. The increase of $OLIG2^{+}$ cells could be happening at the expense of Scl^{+} cells. This expansion is similar to what Muroyama et al.[29] reported in Scl mutants. In this study when Scl is removed, the number of MN progenitors is increased. There is a certain degree of plasticity in these progenitor populations, as V2b progenitors that normally differentiate into V2b INs can give rise to MNs when *Scl* was deleted. When MASH1 is overexpressed, there could be a shift of

progenitors, as the number of MN progenitors (OLIG2$^+$) increases at expense of V2b progenitors (less *Scl*$^+$ cells).

Regarding NGN1 overexpression, we see a repression of *Dll4* and prospective V2b INs while V2a INs are increasing. The number of prospective V2a INs (BHLHB5$^+$LIM3$^+$) also decreases, but the overall number of BHLHB5-positive cells increases, across domains. Taken together, these results point out to a situation where differentiation is accelerated, as more terminally differentiated cells and less prospective INs were detected. NGN1 misexpression might be accelerating differentiation inside the V2 domain, as more fully differentiated CHX10 V2a INs are observed. In fact, proneural proteins have been shown to drive neurogenesis by directing the exit of neural progenitors from the cell cycle and promoting the expression of proteins, specific from post-mitotic neurons [9]. Moreover, proneural proteins capacity to direct neuronal differentiation depends on their ability to suppress *Sox1-3* expression in progenitors [59]. *Sox1-3* maintains neural progenitor cells in an undifferentiated state [59]. Hence, overexpression of NGN1 might be actively repressing *Sox1-3* target genes, and progenitor cells are differentiating prematurely. Furthermore the number of *Dll4*-positive cells, prospective V2a INs (BHLHB5$^+$LIM3$^+$) and prospective V2b INs (*Scl*$^+$) is lower, probably due to the fact that cells already advanced to differentiation (higher number of CHX10$^+$ cells and BHLHB5$^+$ for instance). According to this hypothesis, and as described in the previous section, we were able to detect some CHX10$^+$/CHERRY$^+$ cells, ventrally to the V2 domain, a position where differentiated V2 INs migrate to. Further analysis of both differentiated neurons (*Gata3* and TUJ1) and *Sox1-3* genes should be done in order to validate this hypothesis.

Concerning NGN2 overexpression, we observe that it does not alter the number of prospective V2a INs (bHLHB5$^+$LIM3$^+$) in the electroporated side, however there is a higher number of CHX10$^+$ V2a INs. In this scenario, as a late proneural gene *Ngn2* is most likely just affecting interneuron specification without accelerating differentiation. Once again, NGN2 and NGN1 show different actions, although the two are likely to bind to the same consensus E-box sequences (CANATG). One possible explanation for this fact can rely on the post-translational control of Ngn2 activity [54] or on the fact that NGN2 could need specific co-factors like LMO4 to be transcriptional active. To address this hypothesis, double electroporation of NGN2+LMO4 should be done, and the analysis of *Dll4* expression and prospective V2a (bHLHB5$^+$LIM3$^+$) should be repeated in order to see if NGN2 could mimic NGN1 function.

The molecular mechanisms by which, in the ventral spinal cord, common progenitors differentiate intro different types of interneurons are still poorly unknown. This work provides new data and evidences in terms of neurogenesis inside the V2 domain: the proneural genes *Mash1* and *Ngn1*are acting in earlier steps of cell differentiation, regulating *Dll4* expression and influencing V2 IN specification while *Ngn2* regulates later steps of differentiation, acting on V2 IN specification.

Finally, two different models are proposed for *Mash1* and *Ngn1* function. *Mash1* is keeping progenitors proliferative while *Ngn1* is accelerating neurogenesis.

CHAPTER V

References

CHAPTER V – REFERENCES

1 Gilbert, S. *Developmental Biology*. (2006).

2 Diez del Corral, R. *et al.* Opposing FGF and Retinoid Pathways Control Ventral Neural Pattern, Neuronal Differentiation and Segmentation during Body Axis Extension. *Neuron* **40**, 65-79 (2003).

3 Diez del Corral, R. & Storey, K. G. Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *BioEssays : news and reviews in molecular, cellular and developmental biology* **26**, 857-869, doi:10.1002/bies.20080 (2004).

4 Briscoe, J. & Ericson, J. Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11**, 43-49 (2001).

5 Briscoe, J. & Novitch, B. G. Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences* **363**, 57-70, doi:10.1098/rstb.2006.2012 (2008).

6 Ulloa, F. & Marti, E. Wnt won the war: antagonistic role of Wnt over Shh controls dorso-ventral patterning of the vertebrate neural tube. *Developmental dynamics : an official publication of the American Association of Anatomists* **239**, 69-76, doi:10.1002/dvdy.22058 (2010).

7 Goulding, M. Circuits controlling vertebrate locomotion: moving in a new direction. *Nature reviews. Neuroscience* **10**, 507-518, doi:10.1038/nrn2608 (2009).

8 Guillemot, F. Spatial and temporal specification of neural fates by transcription factor codes. *Development* **134**, 3771-3780, doi:10.1242/dev.006379 (2007).

9 Bertrand, N., Castro, D. S. & Guillemot, F. Proneural genes and the specification of neural cell types. *Nature reviews. Neuroscience* **3**, 517-530, doi:10.1038/nrn874 (2002).

10 Jessell, T. M. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nature reviews. Genetics* **1**, 20-29, doi:10.1038/35049541 (2000).

11 Louvi, A. & Artavanis-Tsakonas, S. Notch signalling in vertebrate neural development. *Nature reviews. Neuroscience* **7**, 93-102, doi:10.1038/nrn1847 (2006).

12 Kopan, R. & Ilagan, M. X. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* **137**, 216-233, doi:10.1016/j.cell.2009.03.045 (2009).

13 Ramos, C., Rocha, S., Gaspar, C. & Henrique, D. Two Notch ligands, Dll1 and Jag1, are differently restricted in their range of action to control neurogenesis in the mammalian spinal cord. *PloS one* **5**, e15515, doi:10.1371/journal.pone.0015515 (2010).

14 Rocha, S. F., Lopes, S. S., Gossler, A. & Henrique, D. Dll1 and Dll4 function sequentially in the retina and pV2 domain of the spinal cord to regulate

neurogenesis and create cell diversity. *Dev Biol* **328**, 54-65, doi:10.1016/j.ydbio.2009.01.011 (2009).

15 Fior, R. *The role of hes genes in the controlled production of neurons* PhD thesis, Universidade de Lisboa, (2006).

16 Bray, S. J. Notch signalling: a simple pathway becomes complex. *Nature reviews. Molecular cell biology* **7**, 678-689, doi:10.1038/nrm2009 (2006).

17 Fior, R. & Henrique, D. A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. *Dev Biol* **281**, 318-333, doi:10.1016/j.ydbio.2005.03.017 (2005).

18 Philpott, A. Neural Development: bHLH Genes. *Encyclopedia of Life Sciences (ELS)*, doi:10.1002/9780470015902.a0000827.pub2 (2010).

19 Parras, C. M., Schuumans, C. & Scardigli, R. Divergent functions of the proneural genes Mash1 and Ngn2 in the specification of neuronal subtype identity. *Genes Development* **16**, 324-338, doi:10.1101/gad.940902 (2002).

20 Turki-Judeh, W. & Courey, A. J. Groucho: a corepressor with instructive roles in development. *Current topics in developmental biology* **98**, 65-96, doi:10.1016/B978-0-12-386499-4.00003-3 (2012).

21 Gotz, M. & Huttner, W. B. The cell biology of neurogenesis. *Nature reviews. Molecular cell biology* **6**, 777-788, doi:10.1038/nrm1739 (2005).

22 Holliday, M. Neurogenesis in the vertebrate neural tube. *Developmental neuroscience* **19**, 161-173 (2001).

23 Henrique, D. *et al.* Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic retina. *Current Biology*, 661-670 (1997).

24 Chitnis, A. The Role of Notch in Lateral Inhibition and Cell Fate Specification. *Molecular and Cellular Neuroscience* (1995).

25 Kageyama, R., Ohtsuka, T., Shimojo, H. & Imayoshi, I. Dynamic Notch signaling in neural progenitor cells and a revised view of lateral inhibition. *Nature neuroscience* **11**, 1247-1251, doi:10.1038/nn.2208 (2008).

26 Castro, D. S. *et al.* Proneural bHLH and Brn proteins coregulate a neurogenic program through cooperative binding to a conserved DNA motif. *Developmental cell* **11**, 831-844, doi:10.1016/j.devcel.2006.10.006 (2006).

27 Wang, S. & Barres, B. A. Up a Notch: Instructing Gliogenesis. *Neuron* **27**, 197-200 (2000).

28 Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. & Weinmaster, G. Expression Patterns of Jagged, Delta1, Notch1, Notch2 and Notch3 Genes Identify Ligand-Receptor Pairs that may fucntion in Neural Development. *Molecular and Cellular Neuroscience* **8**, 14-27 (1996).

29 Muroyama, Y., Fujiwara, Y., Orkin, S. H. & Rowitch, D. H. Specification of astrocytes by bHLH protein SCL in a restricted region of the neural tube. *Nature* **438**, 360-363, doi:10.1038/nature04139 (2005).

30 Peng, C. Y. *et al.* Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. *Neuron* **53**, 813-827, doi:10.1016/j.neuron.2007.02.019 (2007).

31 Benedito, R. & Duarte, A. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. *Gene expression patterns : GEP* **5**, 750-755, doi:10.1016/j.modgep.2005.04.004 (2005).

32 Neves, J. *Regulation of Dll4 expression in cells committing to differentiation in the V2 domain of the developing spinal cord* Master thesis, University of Lisbon, FCUL, (2011).

33 Batista, M. F., Jacobstein, J. & Lewis, K. E. Zebrafish V2 cells develop into excitatory CiD and Notch signalling dependent inhibitory VeLD interneurons. *Developmental Biology* **322**, 263-275, doi:10.1016/j.ydbio.2008.07.015 (2008).

34 Karunaratne, A., Hargrave, M., Poh, A. & Yamada, T. GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev Biol* **249**, 30-43, doi:10.1006/dbio.2002.0754 (2002).

35 Todd, K. J. *et al.* Establishment of motor neuron-V3 interneuron progenitor domain boundary in ventral spinal cord requires groucho-mediated trancriptional corepression. *PloS one* **7**, doi:10.1371/journal.pone.0031176 (2012).

36 Kimura, Y., Satou, C. & Higashijima, S. V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. *Development* **135**, 3001-3005, doi:10.1242/dev.024802 (2008).

37 Lee, S. *et al.* A regulatory network to segregate the identity of neuronal subtypes. *Developmental cell* **14**, 877-889, doi:10.1016/j.devcel.2008.03.021 (2008).

38 Yang, X. *et al.* Notch1 signaling influences v2 interneuron and motor neuron development in the spinal cord. *Developmental neuroscience* **28**, 102-117, doi:10.1159/000090757 (2006).

39 Zhou, Y., Yamamoto, M. & Engel, J. D. GATA2 is required for the generation of V2 interneurons. *Development* **127**, 3829-3838 (2000).

40 Thaler, J. P., Lee, S., Jurata, L. W., Gill, G. N. & Pfaff, S. L. LIM Factor Lhx3 Contributes to the Specification of Motor Neuron and Interneuron Identity through Cell-Type-Specific Protein-Protein Interactions. *Cell* **110**, 237-249 (2002).

41 Sharma, K. *et al.* LIM Homeodomain Factors Lhx3 and Lhx4 Assign Subtype Identities for Motor Neurons. *Cell* **95**, 817-828 (1998).

42 Del Barrio, M. G. *et al.* A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* **134**, 3427-3436, doi:10.1242/dev.005868 (2007).

43 Smith, E., Hargrave, M., Yamada, T., Begley, C. G. & Little, M. H. Coexpression of SCL and GATA3 in the V2 interneurons of the developing mouse spinal cord.

Developmental dynamics : an official publication of the American Association of Anatomists **224**, 231-237, doi:10.1002/dvdy.10093 (2002).

44 Hans, S. & Campos-Ortega, J. A. On the organisation of the regulatory region of the zebrafish deltaD gene. *Development* **129**, 4773-4784 (2002).

45 Li, J. L. *et al.* Delta-like 4 Notch ligand regulates tumor angiogenesis, improves tumor vascular function, and promotes tumor growth in vivo. *Cancer research* **67**, 11244-11253, doi:10.1158/0008-5472.CAN-07-0969 (2007).

46 Sacilotto, N. *et al.* Analysis of Dll4 regulation reveals a combinatorial role for Sox and Notch in arterial development. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 11893-11898, doi:10.1073/pnas.1300805110 (2013).

47 Boije, H., Shirazi Fard, S., Ring, H. & Hallbook, F. Forkheadbox N4 (FoxN4) triggers context-dependent differentiation in the developing chick retina and neural tube. *Differentiation; research in biological diversity* **85**, 11-19, doi:10.1016/j.diff.2012.12.002 (2013).

48 Li, S., Misra, K., Matise, M. P. & Xiang, M. Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 10688-10693, doi:10.1073/pnas.0504799102 (2005).

49 Marklund, U. *et al.* Domain-specific control of neurogenesis achieved through patterned regulation of Notch ligand expression. *Development* **137**, 437-445, doi:10.1242/dev.036806 (2010).

50 Thomas, G. B. & van Meyel, D. J. The glycosyltransferase Fringe promotes Delta-Notch signaling between neurons and glia, and is required for subtype-specific glial gene expression. *Development* **134**, 591-600, doi:10.1242/dev.02754 (2007).

51 Bae, S., Bessho, Y., Hojo, M. & Kageyama, R. The bHLH gene Hes6, an inhibitor of Hes1, promotes neuronal differentiation. *Development* **127**, 2933-2943 (2000).

52 Jacob, J. *et al.* Retinoid acid specifies neuronal identity through graded expression of Ascl1. *Current biology : CB* **23**, 412-418, doi:10.1016/j.cub.2013.01.046 (2013).

53 Asprer, J. S. *et al.* LMO4 functions as a co-activator of neurogenin 2 in the developing cortex. *Development* **138**, 2823-2832, doi:10.1242/dev.061879 (2011).

54 Hindley, C. *et al.* Post-translational modification of Ngn2 differentially affects transcription of distinct targets to regulate the balance between progenitor maintenance and differentiation. *Development* **139**, 1718-1723, doi:10.1242/dev.077552 (2012).

55 Ali, F. *et al.* Cell cycle-regulated multi-site phosphorylation of Neurogenin 2 coordinates cell cycling with differentiation during neurogenesis. *Development* **138**, 4267-4277, doi:10.1242/dev.067900 (2011).

56 Castro, D. S. *et al.* A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes & development* **25**, 930-945, doi:10.1101/gad.627811 (2011).

57 Kim, E. J., Ables, J. L., Dickel, L. K., Eisch, A. J. & Johnson, J. E. Ascl1 (Mash1) Defines Cells with Long-Term Neurogenic Potential in Subgranular and Subventricular Zones in Adult Mouse Brain. *PloS one* **6**, doi:10.1371/journal.pone.0018472.g001 (2011).

58 Southall, T. D. & Brand, A. H. Neural stem cell transcriptional networks highlight genes essential for nervous system development. *The EMBO journal* **28**, 3799-3807, doi:10.1038/emboj.2009.309 (2009).

59 Bylund, M., Andersson, E., Novitch, B. G. & Muhr, J. Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nature neuroscience* **6**, 1162-1168, doi:10.1038/nn1131 (2003).

CHAPTER VI

Annexes

ANNEX A – MATERIALS AND METHODS

DNA quantification

One A260 unit corresponds to 50µg/mL of double stranded DNA50. The purity of the nucleic acid preparation was estimated by the ratio between the readings obtained at 260nm and 280nm (pure preparations of DNA show ratio values of 1.8).

Table 2 – Probes synthetized with respective linearization site and RNA polymerase used.

Probe	Linearization site	RNA polymerase
mHes6	EcoRI	T3
mDll4	BamHI	T3
mLnfng	NotI	T7
cScl	XbaI	T7
cChx10	XhoI	T7
cDll4	EcoRI	T7

Antibodies used during the course of this work

Primary antibodies: sheep anti-Chx10 (Exalpha, 1:100), guinea-pig anti-Bhlhb5 (kindly provided by Bennett Novitch, UCLA, 1:200), rabbit anti-Lim3 (Abcam, 1:50), rabbit anti-Cherry (kindly provided by Adriano Henriques, ITQB, 1:500), rabbit anti-Olig2 (Millipore, 1:100), goat anti-Jag1 (Santa Cruz, 1:100).

Secondary antibodies: donkey anti-sheep (Molecular Probes, 1:400), goat anti-rabbit (Molecular Probes, 1:400), goat anti-guinea-pig (Molecular Probes, 1:400), donkey anti-rabbit (Molecular Probes, 1:400) and donkey anti-goat (Molecular Probes, 1:400).

Table 3 – Composition of all solutions used during this project.

Solution	Composition
1x TAE Buffer	40mM Tris, 1mM EDTA, 0,35% glacial acetic acid
Loading Buffer	60% Glycerol (v/v), 10mM EDTA, 0,2% OrangeG (Sigma)
SOB médium	2% Tryptone, 0,5% Yeast extract, 10mM NaCl, 2.5mM KCl
Hybridization Buffer	1x Salts, 50% Deionised formamide, 10% Dextran Sulphate, 1mg/ml rRNA, 1x Denhardt's solution
Washing Solution	1x SSC, 50% Fromamide
SSC	0.3M Sodium citrate, 3M Sodium chloride, pH 7
Blocking Reagent	10% Boehringer Blocking Reagent (BBR) in maleic acid buffer
Antibody Incubator (ISH)	2% blocking reagent and 1% heat-inactivated sheep serum in TBST
TBST ISH	150mM NaCl, 10mM KCl, 50mM Tris pH 7.5, 0.1% Tween-20
Mowiol	0.1% Mowiol® (Calbiochem), 33% glycerol, 0.1M Tris pH 8.5
DAPI	0.15% (w/v) 4',6-diamidino-2-phenylindole
TBST Immunohistochemistry	20mM TrisHCl pH 8, 150mM NaCl, 0.05% Tween-20

ANNEX B – RESULTS

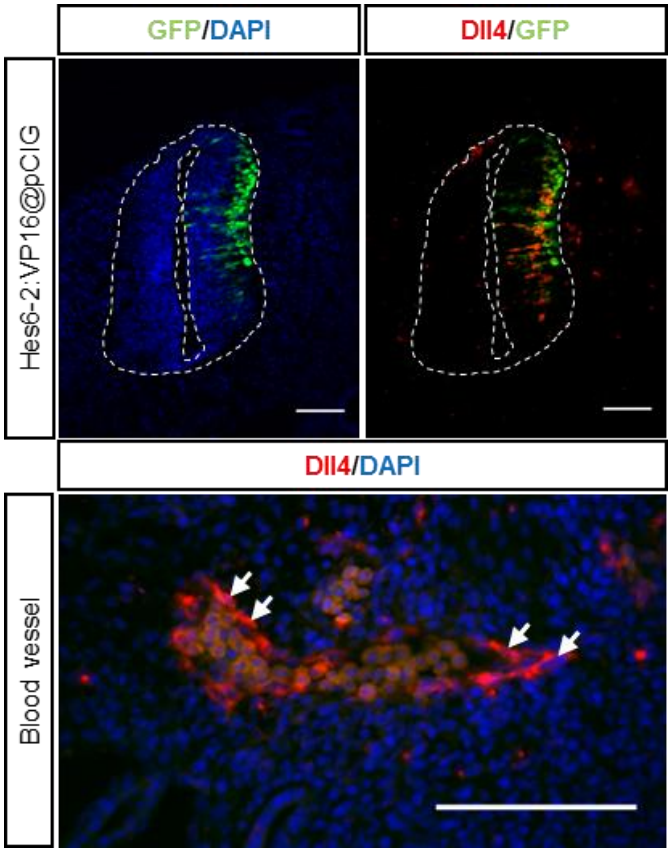

Figure 15 – Controls for Dll4 probe specificity. (A-A') – Hes6-2:VP16 electroporated embryos show Dll4 expression in the electroporated cells, as expected (positive control). (B) – Endogenous endothelial cells marked with Dll4 mRNA probe (white arrows). Scale bars: A-A' – 50µm; B – 20µm